Molecular Design of Multifunctional Food Additives: Antioxidative Antifungal Agents

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A series of alkyl 3,4-dihydroxybenzoates (protocatechuates) was synthesized, and their fungicidal activity against *Saccharomyces cerevisiae* was assayed using a 2-fold serial broth dilution method. Nonyl and octyl 3,4-dihydroxybenzoate were noted to be the most effective against this yeast with the minimum fungicidal concentration of 12.5 μg/mL each. The activity was found to correlate with the hydrophobic alkyl chain length. The time−kill curve study showed that nonyl 3,4-dihydroxybenzoate was fungicidal against *S. cerevisiae* at any growth stage and this activity was not influenced by pH values. The fungicidal activity of alkyl 3,4-dihydroxybenzoates was noted in combination with their ability to disrupt the native membrane-associated function nonspecifically as surface-active agents (surfactants) and to inhibit the respiratory electron transport. However, the primary fungicidal activity of nonyl 3,4-dihydroxybenzoate likely comes from its ability to act as a surfactant.

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 *Saccharomyces 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 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" (soybean pastes). Ethanol is currently permitted for use as a preservative for miso, but it is not cidal against *S. cerevisiae*. Therefore, safe and effective fungicidal agents are still needed.

Recently, we proposed that multifunctional food additives could be designed by selecting appropriate head and tail portions (*2*). For example, geranyl 3,4,5-trihydroxybenzoate (gallate) exhibits antifungal activity against *S. cerevisiae* with a minimum fungicidal concentration (MFC) of 50 μg/mL and acts as an antioxidant (*3*). This benzoate is hydrolyzed to 3,4,5-trihydroxybenzoic acid (gallic acid) and geraniol, and both are common plant components. More importantly, geraniol was previously reported to increase glutathione *S*-transferase activity (*4*), which is believed to be a major mechanism for chemical carcinogen detoxification. The freed 3,4,5-trihydroxybenzoic acid still acts

as an antioxidant (*5−7*), and geraniol induces glutathione *S*-transferase activity. During this experiment, one molecule of 3,4-dihydroxybenzoic acid was found to scavenge six molecules of 1,1-diphenyl-2-picrylhydrazyl (DPPH). In addition, 3,4-dihydroxybenzoic acid and its esters are known to chelate transition metal ions which are powerful promoters of free radical damage in both the human body (*8*) and foods (*9*). Hence, their chelation ability, rendering the metal ions inactive to participate in free radical generating reactions, should be of considerable advantage as antioxidants. On the basis of this concept, a more comprehensive structure−antifungal activity relationship (SAR) study has been carried out. The aim of this paper is to assess the simplest antioxidative antifungal agents against *S. cerevisiae* as an example.

MATERIALS AND METHODS

Chemicals. Geraniol, *trans*-2-nonene-1-ol, 20% Pd(OH)$_2$ on carbon, and *cis*-2-nonene-1-ol were obtained from Alfa Aesar (Ward Hill, MA). 2-Hydroxybenzoic acid, 3-hydroxybenzoic acid, and 4-hydroxybenzoic acid were purchased from Sigma Chemical Co. (St. Louis, MO). 3-Nonanol was purchased from TCI America (Portland, OR). Other primary and secondary alcohols, anethole, 3,4-dihydroxybenzoic acid, 2,3-dihydroxybenzoic acid, 2,4-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, 3,5-dihydroxybenzoic acid, 3-hydroxy-4-methoxybenzoic acid, 4-hydroxy-3-methoxybenzoic acid, 3,4-dimethoxybenzoic acid, tetrahydrofuran (THF), dimethyl sulfoxide (DMSO), *N,N*-dimethylformamide (DMF), DPPH, diisopropyl azodicarboxylate (DIAD), triphenylphosphine (TPP), and *N,N′*-dicyclohexylcarbodiimide (DCC) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Ethanol was purchased from Quantum Chemical Co. (Tuscola, IL).

Synthesis. A series (C$_3$−C$_{14}$) of alkyl 3,4-dihydroxybenzoates were synthesized by one-step esterification utilizing DCC as an activating reagent. In brief, to a solution of 3,4-dihydroxybenzoic acid (1.3 mmol)

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and alcohol (1.3 mmol) in THF (10 mL) cooled at 0 °C was added a solution of DCC (2.0 mmol). After the solution was stirred overnight at room temperature, the solvent was removed under reduced pressure. The residue was extracted with ethyl acetate several times and filtered. The filtrate was washed successively with dilute aqueous citric acid solution, saturated aqueous NaHCO$_3$ solution, and water. The organic layer was dried over Na$_2$SO$_4$ and evaporated. The crude products were purified by silica gel chromatography (10−40% AcOEt−hexane), until urea was completely removed. The best yield (64%) was obtained with butyl 3,4-dihydroxybenzoate. Structures of the synthesized esters were established by spectroscopic methods (IR, MS, and NMR). Because of this synthetically easy accessibility, the construction of a wide range of structurally diverse mimics was made available for evaluation. Log *P* values were calculated by Chem Draw Pro version 4.5 (Cambridge Soft Co., Cambridge, MA) using Crippen's fragmentation (*10*).

Propyl 3,4-dihydroxybenzoate (**1**) was obtained in 55% yield as a colorless powder. ^{1}H NMR (400 MHz, CD$_3$COCD$_3$): δ 1.00 (t, *J* = 7.2 Hz, 3H), 1.75 (sex, *J* = 7.2 Hz, 2H), 4.19 (t, *J* = 7.2 Hz, 2H), 6.91 (d, *J* = 8.0, 1H), 7.46 (dd, *J* = 2.0, 8.0, 1H), 7.52 (d, *J* = 2.0, 1H), 8.50 (bs, 2H). ^{13}C NMR (100 MHz, CD$_3$COCD$_3$): δ 10.8, 22.8, 66.3, 115.6, 116.9, 122.9, 123.0, 145.3, 150.4, 166.3. IR (Nujol): 3500, 3340, 1680, 1610, 1280, 1235, 1100 cm^{-1}. EI-MS: *m/z* 196 (M$^+$).

Butyl 3,4-dihydroxybenzoate (**2**) was obtained in 64% yield as a colorless powder. ^{1}H NMR (400 MHz, CD$_3$COCD$_3$): δ 0.95 (t, *J* = 7.2 Hz, 3H), 1.43 (sex, *J* = 7.2 Hz, 2H), 1.69 (quin, *J* = 7.2 Hz, 2H), 4.22 (t, *J* = 7.2 Hz, 2H), 6.88 (d, *J* = 8.0, 1H), 7.44 (dd, *J* = 2.8, 8.0, 1H), 7.49 (d, *J* = 2.8, 1H), 8.49 (bs, 2H). ^{13}C NMR (100 MHz, CD$_3$COCD$_3$): δ 14.0, 19.9, 31.6, 64.6, 115.6, 116.9, 122.9, 123.0, 145.3, 150.4, 166.3. IR (Nujol): 3490, 3330, 1680, 1610, 1300, 1235, 1100 cm^{-1}. EI-MS: *m/z* 210 (M$^+$).

Pentyl 3,4-dihydroxybenzoate (**3**) was obtained in 51% yield as a colorless powder. ^{1}H NMR (500 MHz, CDCl$_3$): δ 0.92 (t, *J* = 7.0 Hz, 3H), 1.39 (m, 4H), 1.75 (quin, *J* = 7.0 Hz, 2H), 4.28 (t, *J* = 7.0 Hz, 2H), 6.30 (bs, 2H), 6.92 (d, *J* = 8.5, 1H), 7.57 (dd, *J* = 2.0, 8.5, 1H), 7.70 (d, *J* = 2.0, 1H). IR (Nujol): 3500, 3360, 1695, 1615, 1295, 1235, 1100 cm^{-1}. FAB-MS: *m/z* 225 (M + H$^+$).

Hexyl 3,4-dihydroxybenzoate (**4**) was obtained in 33% yield as a colorless powder. ^{1}H NMR (400 MHz, CD$_3$COCD$_3$): δ 0.85 (t, *J* = 6.8 Hz, 3H), 1.29 (m, 4H), 1.40 (m, 2H), 1.68 (quin, *J* = 6.8 Hz, 2H), 4.18 (t, *J* = 6.8 Hz, 2H), 6.85 (d, *J* = 8.0, 1H), 7.41 (dd, *J* = 2.0, 8.0, 1H), 7.46 (d, *J* = 2.0, 1H), 8.45 (bs, 2H). ^{13}C NMR (100 MHz, CD$_3$COCD$_3$): δ 14.3, 23.2, 26.4, 30.6, 32.2, 64.9, 115.6, 116.9, 122.9, 123.0, 145.3, 150.4, 166.3. IR (Nujol): 3510, 3350, 1680, 1610, 1280, 1240, 1100 cm^{-1}. EI-MS: *m/z* 238 (M$^+$).

Heptyl 3,4-dihydroxybenzoate (**5**) was obtained in 35% yield as a colorless powder. ^{1}H NMR (400 MHz, CD$_3$COCD$_3$): δ 0.88 (t, *J* = 6.8 Hz, 3H), 1.32 (m, 8H), 1.72 (quin, *J* = 6.8 Hz, 2H), 4.21 (t, *J* = 6.8 Hz, 2H), 6.89 (d, *J* = 8.4, 1H), 7.44 (dd, *J* = 2.0, 8.4, 1H), 7.50 (d, *J* = 2.0, 1H), 8.50 (bs, 2H). ^{13}C NMR (100 MHz, CD$_3$COCD$_3$): δ 14.3, 23.2, 26.7, 29.5, 29.7, 32.5, 64.9, 115.6, 116.9, 122.9, 123.0, 145.3, 150.4, 166.3. IR (Nujol): 3500, 3340, 1685, 1610, 1300, 1230, 1105 cm^{-1}. EI-MS: *m/z* 252 (M$^+$).

Octyl 3,4-dihydroxybenzoate (**6**) was obtained in 36% yield as a colorless powder. ^{1}H NMR (400 MHz, CD$_3$COCD$_3$): δ 0.83 (t, *J* = 6.8 Hz, 3H), 1.25 (m, 8H), 1.40 (m, 2H), 1.68 (quin, *J* = 6.8 Hz, 2H), 4.18 (t, *J* = 6.8 Hz, 2H), 6.85 (d, *J* = 8.4, 1H), 7.40 (dd, *J* = 2.0, 8.4, 1H), 7.46 (d, *J* = 2.0, 1H), 8.44 (bs, 2H). ^{13}C NMR (100 MHz, CD$_3$COCD$_3$): δ 14.3, 23.3, 26.8, 29.5, 30.0, 30.5, 32.5, 64.9, 115.5, 116.9, 122.9, 123.0, 145.3, 150.4, 166.3. IR (Nujol): 3510, 3350, 1685, 1610, 1300, 1235, 1105 cm^{-1}. EI-MS: *m/z* 266 (M$^+$).

Nonyl 3,4-dihydroxybenzoate (**7**) was obtained in 41% yield as a colorless powder. ^{1}H NMR (500 MHz, CDCl$_3$): δ 0.88 (t, *J* = 7.0 Hz, 3H), 1.27 (m, 10H), 1.42 (m, 2H), 1.74 (quin, *J* = 7.0 Hz, 2H), 4.28 (t, *J* = 7.0 Hz, 2H), 6.33 (bs, 2H), 6.91 (d, *J* = 8.5, 1H), 7.55 (dd, *J* = 2.0, 8.5, 1H), 7.68 (d, *J* = 2.0, 1H). IR (Nujol): 3470, 3330, 1685, 1605, 1285, 1225, 1095 cm^{-1}. FAB-MS: *m/z* 281 (M + H$^+$).

Decyl 3,4-dihydroxybenzoate (**8**) was obtained in 36% yield as a colorless powder. ^{1}H NMR (400 MHz, CD$_3$COCD$_3$): δ 0.86 (t, *J* = 7.2 Hz, 3H), 1.27 (m, 12H), 1.44 (m, 2H), 1.72 (quin, *J* = 6.8 Hz, 2H), 4.22 (t, *J* = 6.8 Hz, 2H), 6.88 (d, *J* = 8.0, 1H), 7.44 (dd, *J* = 2.8,

8.0, 1H), 7.50 (d, *J* = 2.0, 1H), 8.45 (bs, 2H). ^{13}C NMR (100 MHz, CD$_3$COCD$_3$): δ 14.4, 23.3, 26.8, 29.5, 30.2, 32.6, 64.9, 115.5, 116.9, 122.9, 123.0, 145.4, 150.4, 166.3. IR (Nujol): 3460, 3310, 1670, 1605, 1300, 1230, 1100 cm^{-1}. EI-MS: *m/z* 294 (M$^+$).

Undecyl 3,4-dihydroxybenzoate (**9**) was obtained in 50% yield as a colorless powder. ^{1}H NMR (400 MHz, CD$_3$COCD$_3$): δ 0.83 (t, *J* = 7.2 Hz, 3H), 1.24 (m, 16H), 1.69 (quin, *J* = 6.8 Hz, 2H), 4.18 (t, *J* = 6.8 Hz, 2H), 6.85 (d, *J* = 8.0, 1H), 7.41 (dd, *J* = 2.0, 8.0, 1H), 7.46 (d, *J* = 2.0, 1H), 8.43 (bs, 2H). ^{13}C NMR (100 MHz, CD$_3$COCD$_3$): δ 14.4, 23.3, 26.8, 29.5, 30.2, 30.3, 32.6, 64.9, 115.5, 116.9, 122.9, 123.0, 145.3, 150.4, 166.3. IR (Nujol): 3490, 3350, 1695, 1615, 1300, 1235, 1105 cm^{-1}. EI-MS: *m/z* 308 (M$^+$).

Dodecyl 3,4-dihydroxybenzoate (**10**) was obtained in 33% yield as a colorless powder. ^{1}H NMR (400 MHz, CD$_3$COCD$_3$): δ 0.86 (t, *J* = 6.8 Hz, 3H), 1.27 (m, 18H), 1.72 (quin, *J* = 6.8 Hz, 2H), 4.22 (t, *J* = 6.8 Hz, 2H), 6.88 (d, *J* = 8.0, 1H), 7.44 (dd, *J* = 2.0, 8.0, 1H), 7.50 (d, *J* = 2.0, 1H), 8.47 (bs, 2H). ^{13}C NMR (100 MHz, CD$_3$COCD$_3$): δ 14.4, 23.3, 26.8, 29.5, 30.2, 30.3, 32.6, 64.9, 115.5, 116.9, 122.9, 123.0, 145.3, 150.4, 166.3. IR (Nujol): 3480, 3330, 1680, 1615, 1305, 1240, 1105 cm^{-1}. EI-MS: *m/z* 322 (M$^+$).

Tridecyl 3,4-dihydroxybenzoate (**11**) was obtained in 33% yield as a colorless powder. ^{1}H NMR (400 MHz, CD$_3$COCD$_3$): δ 0.86 (t, *J* = 6.8 Hz, 3H), 1.27 (m, 20H), 1.72 (quin, *J* = 6.8 Hz, 2H), 4.21 (t, *J* = 6.8 Hz, 2H), 6.88 (d, *J* = 8.0, 1H), 7.44 (dd, *J* = 2.4, 8.0, 1H), 7.50 (d, *J* = 2.4, 1H), 8.47 (bs, 2H). ^{13}C NMR (100 MHz, CD$_3$COCD$_3$): δ 14.4, 23.3, 26.8, 29.5, 30.0, 30.2, 30.3, 30.4, 32.6, 64.9, 115.5, 116.9, 122.9, 123.0, 145.3, 150.4, 166.3. IR (Nujol): 3490, 3350, 1695, 1615, 1300, 1235, 1100 cm^{-1}. EI-MS: *m/z* 336 (M$^+$).

Tetradecyl 3,4-dihydroxybenzoate (**12**) was obtained in 36% yield as a colorless powder. ^{1}H NMR (400 MHz, CD$_3$COCD$_3$): δ 0.88 (t, *J* = 7.2 Hz, 3H), 1.27 (m, 22H), 1.73 (quin, *J* = 6.8 Hz, 2H), 4.23 (t, *J* = 6.8 Hz, 2H), 6.90 (d, *J* = 8.0, 1H), 7.46 (dd, *J* = 2.0, 8.0, 1H), 7.51 (d, *J* = 2.0, 1H), 8.48 (bs, 2H). ^{13}C NMR (100 MHz, CD$_3$COCD$_3$): δ 14.4, 23.3, 26.8, 29.5, 30.2, 30.3, 30.4, 30.5, 32.6, 64.9, 115.5, 116.9, 122.9, 123.0, 145.3, 150.4, 166.3. IR (Nujol): 3480, 3330, 1680, 1615, 1305, 1240, 1105 cm^{-1}. EI-MS: *m/z* 350 (M$^+$).

Other nonyl benzoates were synthesized as follows (*11*). A mixture of benzyloxybenzoic acid (0.60 mmol) (*12*−*17*), 1-nonanol (0.66 mmol), and TPP (190 mg, 0.72 mmol) in THF (4 mL) was cooled to 0 °C and treated with DIAD (0.72 mmol). After the mixture was stirred for 2 h at room temperature, the solvent was removed in vacuo. The residue was subjected to silica gel chromatography eluted with 1−8% AcOEt−hexane to give an ester as a white solid, which was used in the next step without further purification. The ester was hydrogenated over 20% Pd(OH)$_2$ on carbon (10 mg) in 1% AcOH−AcOEt (4 mL) for 12 h. Filtration through Celite and concentration followed by silica gel chromatography (1−40% AcOEt−hexane) gave pure nonyl benzoate in high yield.

Nonyl 2-hydroxybenzoate (**16**) was obtained in 86% yield as a colorless oil. ^{1}H NMR (500 MHz, CDCl$_3$): δ 0.88 (t, *J* = 7.0 Hz, 3H), 1.29 (m, 8H), 1.36 (m, 2H), 1.44 (quin, *J* = 6.5 Hz, 2H), 1.78 (quin, *J* = 6.5 Hz, 2H), 4.34 (t, *J* = 6.5 Hz, 2H), 6.88 (td, *J* = 1.0, 7.5, 9.0 Hz, 1H), 6.98 (dd, *J* = 1.0, 9.0 Hz, 1H), 7.45 (td, *J* = 2.0, 7.5, 9.0 Hz, 1H), 7.84 (dd, *J* = 2.0, 7.5 Hz, 1H), 10.84 (s, 1H). ^{13}C NMR (125 MHz, CDCl$_3$): δ 14.1, 22.7, 26.0, 28.6, 29.2, 29.5, 31.6, 65.5, 112.7, 117.6, 119.1, 129.9, 135.6, 161.7, 170.2. IR (CCl$_4$): 3180, 2900, 1670, 1475, 1290, 1240, 1200, 1145, 1075 cm^{-1}. EI-MS: *m/z* 264 (M$^+$).

Nonyl 3-hydroxybenzoate (**17**) was obtained in 89% yield as a colorless solid. ^{1}H NMR (500 MHz, CDCl$_3$): δ 0.88 (t, *J* = 7.0 Hz, 3H), 1.29 (m, 8H), 1.35 (m, 2H), 1.43 (quin, *J* = 6.5 Hz, 2H), 1.76 (quin, *J* = 6.5 Hz, 2H), 4.31 (t, *J* = 6.5 Hz, 2H), 5.47 (s, 1H), 7.05 (td, *J* = 1.0, 2.0, 8.0 Hz, 1H), 7.31 (t, *J* = 8.0 Hz, 1H), 7.57 (dd, *J* = 2.0, 2.5 Hz, 1H), 7.61 (td, *J* = 1.0, 2.5, 8.0 Hz, 1H). ^{13}C NMR (125 MHz, CDCl$_3$): δ 14.1, 22.7, 25.0, 26.9, 28.7, 29.2, 29.5, 31.9, 65.4, 116.3, 120.1, 122.0, 129.7, 131.9, 155.7, 166.7. IR (CCl$_4$): 3590, 3420, 2900, 1700, 1680, 1435, 1270, 1200, 1090 cm^{-1}. EI-MS: *m/z* 264 (M$^+$).

Nonyl 4-hydroxybenzoate (**18**) was obtained in 85% yield as a colorless solid. ^{1}H NMR (500 MHz, CDCl$_3$): δ 0.88 (t, *J* = 7.0 Hz, 3H), 1.28 (m, 8H), 1.36 (m, 2H), 1.44 (quin, *J* = 6.5 Hz, 2H), 1.77

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(quin, $J = 6.5$ Hz, 2H), 4.29 (t, $J = 6.5$ Hz, 2H), 5.76 (bs, 1H), 6.87 (dd, $J = 3.0$, 7.0 Hz, 2H), 7.96 (dd, $J = 3.0$, 7.0 Hz, 2H). IR (CCl$_4$): 3600, 3360, 2920, 1700, 1680, 1600, 1150, 1100 cm^{-1}. EI-MS: m/z 264 (M$^+$).

Nonyl 2,3-dihydroxybenzoate (**19**) was obtained in 89% yield as a colorless solid. ^{1}H NMR (500 MHz, CDCl$_3$): δ 0.89 (t, $J = 7.0$ Hz, 3H), 1.29 (m, 8H), 1.36 (m, 2H), 1.44 (quin, $J = 6.7$ Hz, 2H), 1.78 (quin, $J = 6.7$ Hz, 2H), 4.35 (t, $J = 6.7$ Hz, 2H), 5.64 (s, 1H), 6.79 (t, $J = 8.0$ Hz, 1H), 7.10 (dd, $J = 1.5$, 8.0 Hz, 1H), 7.37 (dd, $J = 1.5$, 8.0 Hz, 1H), 11.00 (s, 1H). ^{13}C NMR (125 MHz, CDCl$_3$): δ 14.1, 22.7, 25.9, 28.5, 29.2, 29.5, 31.8, 65.7, 112.7, 119.1, 119.7, 120.5, 145.1, 148.9, 170.4. IR (CCl$_4$): 3550, 3120, 2920, 1660, 1460, 1290, 1250, 1140, 1050 cm^{-1}. EI-MS: m/z 280 (M$^+$).

Nonyl 2,4-dihydroxybenzoate (**20**) was obtained in 88% yield as a colorless solid. ^{1}H NMR (400 MHz, CDCl$_3$): δ 0.87 (t, $J = 7.2$ Hz, 3H), 1.34 (m, 12H), 1.70 (quin, $J = 6.4$ Hz, 2H), 4.27 (t, $J = 6.4$ Hz, 2H), 5.51 (s, 1H), 6.38 (dd, $J = 8.4$, 2.0 Hz, 1H), 6.40 (d, $J = 2.0$ Hz, 1H), 7.76 (d, $J = 8.4$ Hz, 1H), 11.09 (s, 1H). ^{13}C NMR (100 MHz, CDCl$_3$): δ 14.2, 22.8, 26.1, 28.7, 29.3, 29.5, 31.9, 65.3, 103.0, 106.1, 107.6, 131.8, 161.6, 163.4, 169.8. IR (CCl$_4$): 3590, 3400, 3150, 2910, 1655, 1325, 1255, 1130 cm^{-1}. EI-MS: m/z 280 (M$^+$).

Nonyl 2,5-dihydroxybenzoate (**21**) was obtained in 98% yield as a colorless solid. ^{1}H NMR (500 MHz, CDCl$_3$): δ 0.88 (t, $J = 7.0$ Hz, 3H), 1.28 (m, 8H), 1.35 (m, 2H), 1.43 (quin, $J = 6.7$ Hz, 2H), 1.77 (quin, $J = 6.7$ Hz, 2H), 4.33 (t, $J = 6.7$ Hz, 2H), 4.62 (s, 1H), 6.88 (d, $J = 8.9$ Hz, 1H), 7.00 (dd, $J = 3.0$, 8.9 Hz, 1H), 7.29 (d, $J = 3.0$ Hz, 1H), 10.41 (s, 1H). ^{13}C NMR (125 MHz, CDCl$_3$): δ 14.1, 22.7, 26.0, 28.6, 29.3, 29.5, 31.9, 65.6, 112.5, 114.7, 118.5, 123.8, 147.6, 156.0, 169.8. IR (CCl$_4$): 3610, 3220, 2920, 1670, 1480, 1300, 1210, 1070 cm^{-1}. EI-MS: m/z 280 (M$^+$).

Nonyl 3,5-dihydroxybenzoate (**22**) was obtained in 90% yield as a colorless solid. ^{1}H NMR (750 MHz, CDCl$_3$): δ 0.87 (t, $J = 6.8$ Hz, 3H), 1.32 (m, 8H), 1.37 (m, 2H), 1.45 (quin, $J = 6.8$ Hz, 2H), 1.77 (quin, $J = 6.8$ Hz, 2H), 4.31 (t, $J = 6.8$ Hz, 2H), 5.20 (bs, 2H), 6.59 (m, 1H), 7.13 (d, $J = 1.5$ Hz, 2H). IR (CCl$_4$): 3350, 2910, 1680, 1590, 1330, 1240, 1160 cm^{-1}. EI-MS: m/z 280 (M$^+$).

Nonyl 3-hydroxy-4-methoxybenzoate (**23**) was obtained in 93% yield as a colorless solid. ^{1}H NMR (500 MHz, CDCl$_3$): δ 0.88 (t, $J = 7.3$ Hz, 3H), 1.28 (m, 8H), 1.35 (m, 2H), 1.43 (quin, $J = 6.4$ Hz, 2H), 1.76 (quin, $J = 6.4$ Hz, 2H), 3.92 (s, 3H), 4.27 (t, $J = 6.4$ Hz, 2H), 5.63 (s, 1H), 6.87 (d, $J = 8.5$ Hz, 1H), 7.59 (d, $J = 2.0$ Hz, 1H), 7.62 (dd, $J = 2.0$, 8.5 Hz, 1H). ^{13}C NMR (125 MHz, CDCl$_3$): δ 14.1, 22.7, 26.1, 28,8, 29.3, 29.5, 31.9, 56.0, 65.0, 109.8, 115.6, 122.7, 123.9, 145.2, 150.3, 166.3. IR (CCl$_4$): 3550, 2920, 1710, 1500, 1270, 1200, 1110 cm^{-1}. EI-MS: m/z 294 (M$^+$).

Nonyl 4-hydroxy-3-methoxybenzoate (**24**) was obtained in 91% yield as a colorless solid. ^{1}H NMR (500 MHz, CDCl$_3$): δ 0.87 (t, $J = 7.0$ Hz, 3H), 1.28 (m, 8H), 1.36 (m, 2H), 1.42 (quin, $J = 6.7$ Hz, 2H), 1.77 (quin, $J = 6.7$ Hz, 2H), 3.95 (s, 3H), 4.29 (t, $J = 6.7$ Hz, 2H), 6.00 (s, 1H), 6.94 (d, $J = 8.2$ Hz, 1H), 7.55 (d, $J = 2.0$ Hz, 1H), 7.63 (dd, $J = 2.0$, 8.2 Hz, 1H). ^{13}C NMR (125 MHz, CDCl$_3$): δ 14.1, 22.7, 26.0, 28,6, 29.4, 29.6, 31.9, 56.1, 65.0, 111.7, 114.0, 122.7, 124.1, 145.1, 150.0, 166.4. IR (CCl$_4$): 3540, 2920, 1700, 1500, 1420, 1270, 1210, 1090 cm^{-1}. EI-MS: m/z 294 (M$^+$).

Nonyl 3,4-dimethoxybenzoate (**25**) was obtained in 78% yield as a colorless solid. ^{1}H NMR (500 MHz, CDCl$_3$): δ 0.88 (t, $J = 7.0$ Hz, 3H), 1.29 (m, 8H), 1.36 (m, 2H), 1.44 (quin, $J = 6.5$ Hz, 2H), 1.76 (quin, $J = 6.5$ Hz, 2H), 3.93 (s, 6H), 4.29 (t, $J = 6.5$ Hz, 2H), 6.88 (d, $J = 8.5$ Hz, 1H), 7.55 (d, $J = 2.0$ Hz, 1H), 7.68 (dd, $J = 2.0$, 8.5 Hz, 1H). IR (CCl$_4$): 2920, 1700, 1500, 1450, 1400, 1260, 1210, 1170 cm^{-1}. EI-MS: m/z 308 (M$^+$).

Test Strain and Medium. The test strain, *S. cerevisiae* ATCC 7754, used for this study was purchased from American Type Culture Collection (Manassas, VA). The strain was maintained at −80 °C in yeast nitrogen broth (Yeast Nitrogen Base; Difco, BD, Franklin Lakes, NJ) and subcultured at 30 °C in Sabouraud's dextrose agar medium (Bactopeptone 1%, dextrose 4%, Bacto agar 1.8%). A fresh culture was statically preincubated for 16 h at 30 °C in 2.5% malt extract (ME) medium (BD).

Antifungal Assay. The minimum inhibitory concentration (MIC) was determined using a serial broth dilution method as previously described (*19*). The yeast cells at 10^5 colony formatting unit (CFU)/ mL were statically cultivated in 3 mL of 2.5% ME medium containing 30 μL of DMF with each drug at 30 °C for 48 h. The MIC is defined as the lowest concentration of test compound that showed no visible growth. The MFCs were examined as follows. After the MIC was determined, a 30 μL aliquot was withdrawn from each culture and added into 3 mL of a drug-free fresh ME medium. After a 48-h incubation, the MFC was determined as the lowest concentration of test compounds in which no recovery of microorganism was observed.

Time−Kill Studies. Time−kill studies were performed to examine the effects of compounds in more detail. A 30 μL aliquot of the seed culture was inoculated into 3 mL of ME medium containing appropriate concentrations of the test compounds. The initial population size for *S. cerevisiae* was 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 (1% yeast extract, 2% Bacto peptone, 2% dextrose, 2% agar) plates. The plates were incubated at 30 °C for 48 h before the number of CFUs was determined.

Combination Studies. The combination data were obtained by the broth checkerboard (*20, 21*). A series of 2-fold dilutions of anethole were tested in combination with concentrations of 2-fold dilutions of nonyl dihydroxybenzoate. After incubation at 30 °C for 48 h, the MICs were determined by using the method aforementioned.

Medium Acidification Assay. The inhibitory effect of nonyl 3,4-dihydroxybenzoate on the glucose-induced medium acidification by the plasma-membrane H$^+$-ATPase of *S. cerevisiae* was measured with a modified procedure based on a method described in the literature (*22*). The precultured yeast cells were washed twice with chilled distilled water and then kept on ice. The cell suspensions (10^8 CFU/mL) with the compounds being tested were preincubated at 30 °C for 5 min. The medium acidification was started by addition of 2% D-glucose. After another 10-min incubation, the pH in the external medium was checked.

Adsorption Test. The test strain was cultured with shaking in YPD medium overnight at 30 °C and washed twice with 50 mM MOPS buffer (pH 6.0). After each alkyl protocatechuate (50, 25, 12.5, 6.25, and 3.13 μg/mL, respectively) was mixed with or without *S. cerevisiae* cells (10^8 CFU/mL) in the above buffer at 30 °C, the suspension was vortexed for 2 s. Absorbance of the supernatants obtained by centrifugation at 4000*g* for 5 min was measured at 300 nm.

Efflux of Potassium Ions from Yeast Cells. The precultured yeast cells were washed and then suspended in 67 mM succinate buffer (pH 6.0) to give 10^8 cells/mL. After a 10-min preincubation, nonyl 3,4-dihydroxybenzoate was added and then cell suspensions were incubated at 30 °C. Portions of the suspension were withdrawn. The supernatants obtained by centrifugation at 4000*g* for 5 min from the suspensions were submitted to potassium ion determinations by flame photometry (*23*). Cell viability was also checked by the methylene blue method (*24*).

Measurement of Cellular Leakage. Cell leakage was assessed by measuring 260 nm absorbing materials released to the medium, primarily representing nucleotides of which uracil-containing compounds exhibited the strongest absorbance. One milliliter of 67 mM succinate buffer (pH 6.0) including nonyl 3,4-dihydroxybenzoate with washed yeast cells (10^8 cells) was incubated at 30 °C for 5 min. After incubation, UV-absorbing materials in the supernatant by centrifugation (4000*g* for 5 min) were quantified at 260 nm.

Radical Scavenging Activity on DPPH. First, 1 mL of 100 mM acetate buffer (pH 5.5), 1.87 mL of ethanol, and 0.1 mL of ethanolic solution of 3 mM DPPH were put into a cuvette. Then, 0.03 mL of the sample DMSO solution was added to the cuvette and the decay of absorbance was measured immediately at 517 nm ($\epsilon = 8320$). As a control, 0.03 mL of DMSO was added to the cuvette. From the decrease of the absorbance, scavenging activity was calculated and expressed as scavenged DPPH molecules per molecule.

Data Analysis and Curve Fitting. The assays were performed at least in triplicate on separate occasions. The experimental data were delineated and analyzed by using Sigma Plot 2000 (SPSS Inc., Chicago, IL).
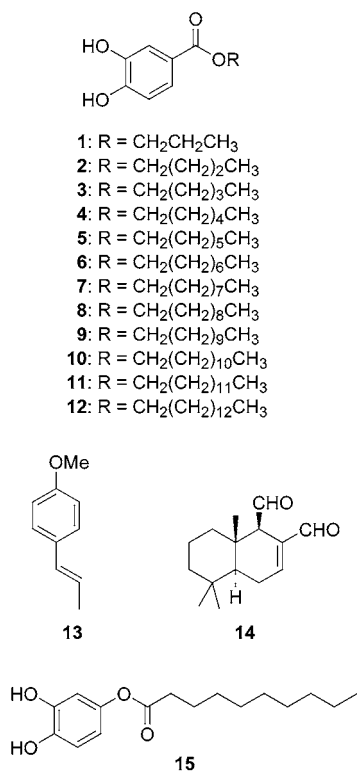
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Figure 1. Chemical structures of alkyl 3,4-dihydroxybenzoates.

1: R = CH₂CH₂CH₃
2: R = CH₂(CH₂)₂CH₃
3: R = CH₂(CH₂)₃CH₃
4: R = CH₂(CH₂)₄CH₃
5: R = CH₂(CH₂)₅CH₃
6: R = CH₂(CH₂)₆CH₃
7: R = CH₂(CH₂)₇CH₃
8: R = CH₂(CH₂)₈CH₃
9: R = CH₂(CH₂)₉CH₃
10: R = CH₂(CH₂)₁₀CH₃
11: R = CH₂(CH₂)₁₁CH₃
12: R = CH₂(CH₂)₁₂CH₃

Table 1. Antifungal Activity of Alkyl 3,4-Dihydroxybenzoate against *S. cerevisiae* ATCC 7754

compound tested	MIC (*MFC*)[a] (μg/mL)	log *P*
C_3	>400 (*>400*)	1.90
C_4	200 (*400*)	2.32
C_5	100 (*100*)	2.73
C_6	25 (*25*)	3.15
C_7	12.5 (*12.5*)	3.57
C_8	6.25 (*12.5*)	3.99
C_9	6.25 (*12.5*)	4.40
C_{10}	>400 (*>400*)	4.82
C_{11}	>400 (*>400*)	5.24
miconazole	6.25 (*50*)	

[a] Numbers in italic type in parentheses are MFCs. Log *P* values were calculated by the method described (*10*).

RESULTS AND DISCUSSION

Fungicidal Activity. A homologous series of alkyl 3,4-dihydroxybenzoates (protocatechuates) was synthesized (see **Figure 1** for structures) and tested for their fungicidal activity against *S. cerevisiae* ATCC 7754 using a 2-fold serial broth dilution method. Since the head portion of all the compounds tested is the same, the data are interpreted to mean that changes in the hydrophobic portion of these 3,4-dihydroxybenzoates correlated to their mode of antifungal action. The results are listed in **Table 1**. The potency of alkyl 3,4-dihydroxybenzoates is noted to be nearly comparable with those of the corresponding alkyl gallates (3,4,5-trihydroxybenzoates) (*2*), indicating that the additional hydroxyl group is not essential. In general, *S. cerevisiae* showed different susceptibilities to alkyl 3,4-dihydroxybenzoates possessing different chain lengths, similar to those found for alkyl gallates (*2*). The range of the antifungal activity of the 3,4-dihydroxybenzoates tested against *S. cerevisiae* is between 12.5 and 400 μg/mL. The cutoff phenomenon in the growth inhibition (MIC) and the fungicidal (MFC) effect was observed between nonyl (C_9) and decyl (C_{10}) 3,4-dihy-
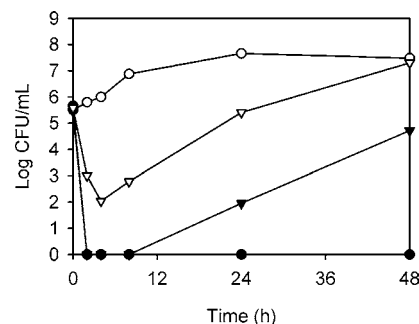

Figure 2. Effect of nonyl protocatechuate on the growth of *S. cerevisiae* ATCC 7754. Exponentially growing cells were inoculated into ME medium and then cultured at 30 °C without shaking. Nonyl 3,4-dihydroxybenzoate: 0 (○), 3.13 (▽), 6.25 (▼), and 12.5 (●) μg/mL.

droxybenzoates. The maximum potency of both the growth inhibition and the fungicidal effect was found in octyl (C_8) and nonyl (C_9) 3,4-dihydroxybenzoates each at 6.25 and 12.5 μg/mL, respectively. The differences between the MIC and MFC values are not more than 2-fold, suggesting that their activity is fungicidal.

The fungicidal effect of nonyl 3,4-dihydroxybenzoate against *S. cerevisiae* was confirmed by time−kill curve experiments. This method measures viable counts over time of microbial colonies plated on agar medium. The cultures of *S. cerevisiae*, with a cell density of 10^5 CFU/mL, were exposed to three different concentrations of nonyl 3,4-dihydroxybenzoate, 1/2MIC, MIC, and 2MIC (equivalent to MFC). The results are illustrated in **Figure 2**. The number of viable cells was determined following different periods of incubation with nonyl 3,4-dihydroxybenzoate. The result of the assay shows that MIC slowed growth but the final cell count recovered up to 10^5 CFU/mL. Complete lethality occurred at 12.5 μg/mL (equivalent to MFC) within 2 h, and no viable cells were recovered up to 48 h. This rapid lethality very likely indicates that the antifungal activity of nonyl 3,4-dihydroxybenzoate against *S. cerevisiae* is associated with the disruption of the membrane, similar to nonyl gallate (*25*). In addition, the fungicidal effect of hexyl 3,4-dihydroxybenzoate against *S. cerevisiae* was also confirmed by the time−kill curve method (data not illustrated).

Subsequently, cultures of *S. cerevisiae* were exposed to nonyl 3,4-dihydroxybenzoate after 8 h (the exponentially growing stage) or 24 h (the stationary growing stage) of incubation. The result shows that nonyl 3,4-dihydroxybenzoate at 12.5 μg/mL (MFC) or 25 μg/mL (2MFC) was not fungicidal when added to both the exponentially (10^7 CFU/mL) and stationary (10^8 CFU/mL) growing culture stages. The viable cells were rapidly reduced but recovered slowly. As shown in **Figure 3**, complete lethality occurred after being exposed to 50 μg/mL (equivalent to 4MFC) of nonyl 3,4-dihydroxybenzoate when added to the exponentially growing culture stage. No viable cells were recovered up to 48 h. However, nonyl 3,4-dihydroxybenzoate was not fungicidal when added to the stationary culture stage. In the latter case, the viable cells were reduced down to 10^3 CFU/mL within 2 h but the final cell count was not significantly different from this as shown in **Figure 3**. The result obtained indicates that nonyl 3,4-dihydroxybenzoate is fungicidal against *S. cerevisiae* at the exponentially growing stage but not at the stationary growing stage. Subsequently, the cells (10^7 CFU/mL) obtained after 24 h of precultivation of the seed culture were diluted 100-fold, and the resulting cells (10^5 CFU/mL) were subjected to the antifungal activity. The data obtained were the same as for alkanols, indicating that the antifungal action of nonyl 3,4-dihydroxybenzoate is dependent on cell density but
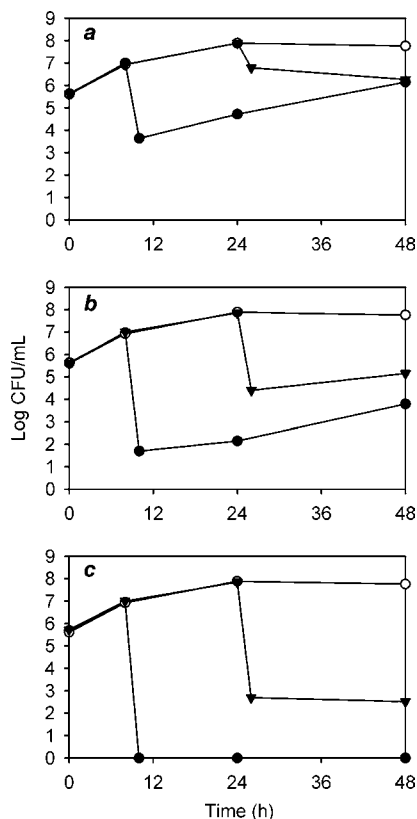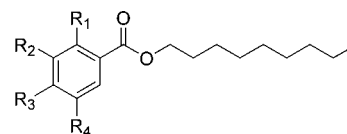
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Figure 3. Effect of growth phase on the nonyl 3,4-dihydroxybenzoic acid induced cell death of *S. cerevisiae* ATCC 7754. The compound was added at (a) 12.5, (b) 25, or (c) 50 μg/mL after 8 h (●) or 24 h (▼) of cultivation. Control (○) indicates no addition of the compound.

16: R$_1$ = OH, R$_2$ = R$_3$ = R$_4$ = H
17: R$_1$ = R$_3$ = R$_4$ = H, R$_2$ = OH
18: R$_1$ = R$_2$ = R$_4$ = H, R$_3$ = OH
19: R$_1$ = R$_2$ = OH, R$_3$ = R$_4$ = H
20: R$_1$ = R$_3$ = OH, R$_2$ = R$_4$ = H
21: R$_1$ = R$_4$ = OH, R$_2$ = R$_3$ = H
22: R$_1$ = R$_3$ = H, R$_2$ = R$_4$ = OH
23: R$_1$ = R$_4$ = H, R$_2$ = OH, R$_3$ = OMe
24: R$_1$ = R$_4$ = H, R$_2$ = OMe, R$_4$ = OH
25: R$_1$ = R$_4$ = H, R$_2$ = R$_3$ = OMe

Figure 4. Chemical structures of synthetic nonyl hydroxybenzoates.

not growth stage. In addition, the time−kill experiment was performed with hexyl 3,4-dihydroxybenzoate against *S. cerevisiae* and similar results were obtained. However, hexyl 3,4-dihydroxybenzoate is fungicidal at both exponentially and stationary growing stages after the addition of 100 μg/mL (equivalent to 4MFC) of this dihydrobenzoate.

The antimicrobial activity of alkyl 3,4-dihydroxybenzoates is nonspecific (data not listed), and the potency of the activity against *S. cerevisiae* was distinctly increased with each additional CH$_2$ group up to octyl 3,4-dihydroxybenzoate. In the time−kill experiment of nonyl 3,4-dihydroxybenzoate against *S. cerevisiae*, lethality occurred notably quickly, within the first 2 h after the addition of nonyl 3,4-dihydroxybenzoate, and fungicidal activity was found at any growth stage. Taking altogether, the antifungal activity of amphipathic alkyl 3,4-dihydroxybenzoates against *S. cerevisiae* is mediated primarily due to its nonionic surfactant property, similar to those described for amphipathic alkyl gallates (*26*).

In the case against *S. cerevisiae*, a common phenyl propanoid, anethole (**13**), is known to significantly enhance the fungicidal activity of other antifungal compounds such as polygodial (**14**) and sorbic acid (*27, 28*). Hence, anethole was combined with nonyl 3,4-dihydroxybenzoate to see if the combination has any enhancing activity against *S. cerevisiae*, but the results did not show any noticeable enhancing activity. The combination was only additive.

Structural Criteria. The "hydrolyzable" ester group was selected in order to avoid undesired side effects, particularly endocrine-disrupting activity of environmentally persistent estrogen mimics (*29*) such as alkylphenolic compounds (*30*). The esters were synthesized mainly by one-step esterification as described above. Because of this synthetically easy acces-

sibility, the construction of a wide range of structurally diverse mimics was also made available for comparison. In addition, the ester group is not directly related to the activity since 3,4-dihydroxyphenyl decanoate (**15**) exhibited a fungicidal activity against *S. cerevisiae* comparable to that of nonyl 3,4-dihydroxybenzoate (*11*). If *S. cerevisiae* cells do secrete nonspecific extracellular esterases that hydrolyze nonyl 3,4-dihydroxybenzoate to 3,4-dihydroxybenzoic acid and 1-nonanol, the antifungal activity observed could be expected from the hydrolysates. This can be ruled out since the MFC of 1-nonanol against *S. cerevisiae* ATCC 7754 was 200 μg/ mL and 3,4-dihydroxybenzoic acid did not exhibit any fungicidal activity against *S. cerevisiae* up to 3200 μg/mL.

As long as the head and tail structure is balanced, both hydrophilic and hydrophobic portions seems to be flexible. Since nonyl 3,4-dihydroxybenzoate was found to be the most effective against *S. cerevisiae*, similar to results found for alkyl gallates (*31*), various nonyl phenylbenzoates such as nonyl 2-hydroxybenzoate (**16**), nonyl 3-hydroxybenzoate (**17**), nonyl 4-hydroxybenzoate (**18**), nonyl 2,3-dihydroxybenzoate (**19**), nonyl 2,4-dihydroxybenzoate (**20**), and nonyl 2,5-dihydroxybenzoate (**21**) were synthesized and tested for their antifungal activity against *S. cerevisiae* for comparison (see **Figure 4** for structures). None of the nonyl monohydroxybenzoates tested (**16**−**18**) showed any antifungal activity. Notably, nonyl 3,5-dihydroxybenzoate (**22**) was the only active compound. It appears therefore that two hydroxyl groups are essential to elicit the activity as the hydrophilic "head" portion. This can be supported by the observation that neither nonyl 3-hydroxy-4-methoxybenzoate (**23**) nor nonyl 4-hydroxy-3-methoxybenzoate (**24**) showed any antifungal activity. Interestingly, none of the compounds nonyl 2,3-dihydroxybenzoate, nonyl 2,4-dihydroxybenzoate, nonyl 2,5-dihydroxybenzoate, and nonyl 3,4-dimethoxybenzoate (**25**) exhibited any antifungal activity. In brief, none of these salicylic acid derivatives (**16**, **20**, **21**) exhibited any antifungal activity. A small change in chemical structures, especially in the head portion, was noted to affect biological activity to a large extent. Amphipathic alkyl hydroxybenzoates may have the potential to be fungicidal agents since they very likely target the extracytoplasmic region and thus do not need to enter into the cell, thereby avoiding most cellular pump-based resistance mechanisms.

The head and tail structure and nonspecific antimicrobial activity of alkyl 3,4-dihydroxybenzoates are similar to those
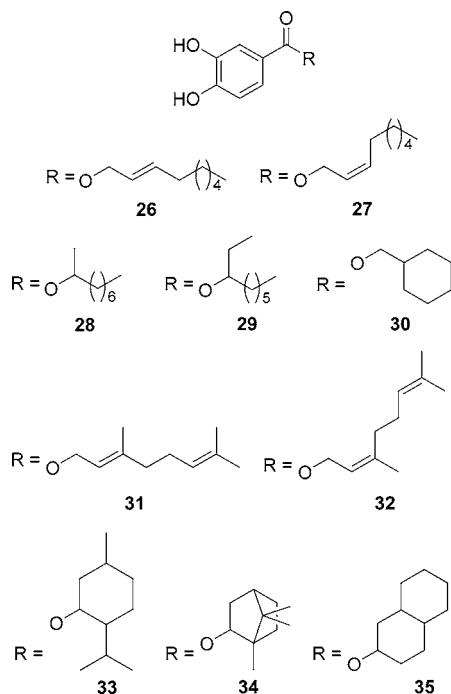
Figure 5. Chemical structures of bulky alk(en)yl 3,4-dihydroxybenzoates.

Table 2. Antifungal Activity of Bulky Alkyl 3,4-Dihydroxybenzoates against *S. cerevisiae* ATCC 7754

compound tested	MIC (*MFC*) (µg/mL)[a]	log *P*
trans-2-nonene-1-yl (**26**)	6.25 (*6.25*)	4.22
cis-2-nonene-1-yl (**27**)	6.25 (*6.25*)	4.22
2-nonanyl (**28**)	12.5 (*12.5*)	4.30
3-nonanyl (**29**)	25 (*50*)	4.37
cyclohexylmethyl (**30**)	50 (*50*)	3.05
geranyl (**31**)	12.5 (*12.5*)	3.84
neryl (**32**)	12.5 (*25*)	3.84
menthyl (**33**)	12.5 (*12.5*)	4.10
bornyl (**34**)	50 (*50*)	3.78
decahydro-2-naphthyl (**35**)	50 (*50*)	3.62

[a] Numbers in italic type in parentheses are MFCs.

found for alkanols to some extent (*19*, *32*). The hydrophilic head part binds with an intermolecular hydrogen bond like a "hook" attaching itself to the hydrophilic portion of the membrane surface. Subsequently, the hydrophobic tail portion of the molecule is able to enter into the membrane lipid bilayer. This creates, as a result, disorder in the fluid bilayer of the membrane. Although the binding site remains unclear, the two hydroxyl groups are established as the hook in certain amphipathic alkyl hydroxybenzoates. Compared to alkanols, a top-heavy structure of fungicidal hydroxybenzoates likely needs at least two hydroxyl groups to form a stable intermolecular hydrogen bond with the membrane surface, allowing the alkyl tail to align in the hydrophobic portion of the membrane by dispersion forces. In the case of nonyl 2,3-, 2,4-, and 2,5-dihydrobenzoates, the salicylate moiety of ***group a*** is in a sterically crowded environment and is forming an intramolecular hydrogen bond (**Figure 4**). This may suggest that the moiety of ***group a*** is not able to bind the membrane surface of *S. cerevisiae* and hence cannot act as a surfactant.

The length, unsaturation, and volume of the hydrophobic alkyl group are associated with their fungicidal activity, and branched-chain or ring-containing surfactants are generally more soluble in aqueous media than straight-chain materials with the same number of carbon atoms (*33*). Since the hydrophobic portion of the molecule enters into the membrane lipid bilayer and creates disorder in the fluid bilayer, increasing the volume of the hydrophobic portion through synthetic modification may enhance the activity. On the basis of this concept, various bulky alkyl 3,4-dihydroxybenzoates, *trans*-2-nonene-1-yl (**26**), *cis*-2-nonene-1-yl (**27**), 2-nonanyl (**28**), 3-nonanyl (**29**), cyclohexylmethyl (**30**), geranyl (**31**), neryl (**32**), menthyl (**33**), bornyl (**34**), and decahydro-2-naphthyl (**35**), were synthesized in the same manner (*3*) and assayed against the same *S. cerevisiae* strain for comparison (see **Figure 5** for structures). The results are listed in **Table 2**. The compounds possessing similar log *P* values exhibit similar activity, and 6.25 µg/mL seems to be the maximum activity through synthetic optimization. Overall, the molecular shape does not appear to be a major contributor to the activity. Additional biological activities can also be intro-

duced by selecting appropriate hydrophobic moieties. For example, geraniol has previously been reported to increase glutathione *S*-transferase activity, which is believed to be a major mechanism for chemical carcinogen detoxification (*4*). The antioxidant 3,4-dihydroxybenzoic acid and the glutathione *S*-transferase inducer geraniol may contribute to reduce cancer risk as well as oxidative-damage-related diseases.

Mode of Fungicidal Action. The addition of glucose to an unbuffered suspension of *S. cerevisiae* cells results in the extrusion of acid. The change in external pH upon the addition of glucose is characteristic of yeast cells. This acid extruded could be due to the action of the plasma membrane H$^+$-ATPase (*34*). The activation of the H$^+$-ATPase by glucose is not yet fully understood on a molecular basis, but the maintenance of internal pH homeostasis is essential for the cell to survive since intracellular pH is important for the activity of a number of enzymes with pH optima (*35*). This glucose-induced medium acidification process was inhibited by nonyl 3,4-dihydroxybenzoate. The inhibition was presumably caused by its inhibition of the H$^+$-ATPase. (*36*). It seems that nonyl 3,4-dihydroxybenzoate inhibited this plasma membrane transporter and other membrane-bound proteins as a nonionic surfactant, similar to amphipathic nonyl gallate (*25*).

Leakage of K$^+$ is a primary indication of membrane damage (*37*). However, it is difficult to distinguish whether leakage of K$^+$ ion is caused by plasma membrane damage or energy-dependent positive efflux of the ion due to drug stress because yeast cells are known to activate efflux of K$^+$ ion in the presence of glucose to maintain intracellular content of the ion (*38*). On the other hand, no efflux of K$^+$ ion has been described in the absence of energy sources such as glucose (*38*). In the current experiments, leakage tests were performed in buffer without any energy sources. The leakage of K$^+$ ion by nonyl 3,4-dihydroxybenzoate was observed within 30 min of treatment accompanied with significant loss of cell viability. However, nonyl 3,4-dihydroxybenzoate did not induce the leakage of 260 nm absorbing materials. The plasma membrane damage probably resulted in leakage of K$^+$ ion to the buffer, indicating that the rapid decrease in the intracellular level of K$^+$ ion by nonyl 3,4-dihydroxybenzoate could induce cell death by plasma membrane damage. These results suggest that nonyl 3,4-dihydroxybenzoate quickly affects the plasma membrane of yeast cells forming rather smaller size pores that permitting transit of K$^+$ ion but not 260 nm absorbing materials, similar to those found for nonyl gallate (*25*).

Further support for the surfactant concept was obtained in an additional experiment that indicates fungicidal nonyl proto-catechuate rapidly adsorbed onto the surface of *S. cerevisiae* cells but propyl and dodecyl 3,4-dihydroxybenzoates did
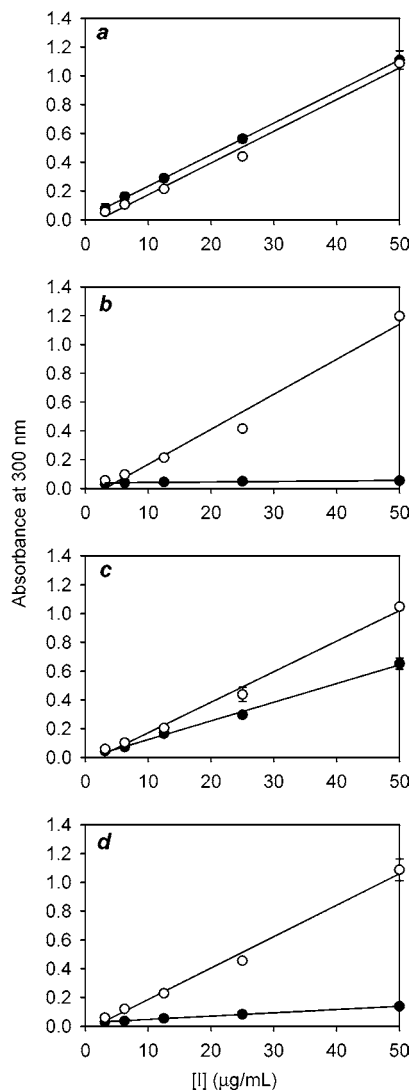
Figure 6. Adsorption of propyl (**a**), nonyl (**b**), dodecyl (**c**), and menthyl (**d**) protocatechuates to the cells of *S. cerevisiae* ATCC 7754. After each protocatechuate was mixed with (●) or without (○) *S. cerevisiae* cells (10^8 cells/mL), the suspension was vortexed for 2 s. Absorbance of the supernatant obtained by centrifugation for 5 min was measured. Each plot is the mean of triplicate determinations. The data were fitted with the linear equation (each $R^2 \geq 0.99$).

slightly, as shown in **Figure 6**. The hydrophilic head portion adsorbed by an intermolecular hydrogen bond in attaching itself to the hydrophilic portion of the membrane surface. It appears that *S. cerevisiae* showed different affinities to 3,4-dihydroxybenzoates having different alkyl chain lengths. The hydrophilic portion adsorbed by an intermolecular hydrogen bond in attaching itself to the hydrophilic portion of the membrane surface. The adsorbing sites may not be specific but need to be clarified. On the other hand, most of the dodecyl 3,4-dihydroxybenzoate molecules did not adsorb onto the cell surface and remained in the water-based test medium, probably as an insoluble monolayer or spread film (*39*). As expected, menthyl 3,4-dihydroxybenzoate rapidly adsorbed onto the surface of *S. cerevisiae* cells and this benzoate exhibited a similar antifungal activity with an MFC of 12.5 μg/mL. It seems that the log *P* value is related to *S. cerevisiae* cell surface hydrophobicity (*40*).

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 nonfermentable 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. Nonyl 3,4-dihydroxybenzoate also inhibits the growth of *S. cerevisiae* growing on nonfermentable carbon sources such as ethanol-, lactate-, acetate-, and glycerol-containing media. As a surfactant, nonyl 3,4-dihydroxybenzoate exhibits fungicidal activity when *S. cerevisiae* is growing in both fermentable and nonfermentable conditions.

In addition, alkyl 3,4-dihydroxybenzoates were found to inhibit bacterial respiratory systems. For example, both nonyl and dodecyl 3,4-dihydroxybenzoates inhibited the oxygen consumption of *Pseudomonas aeruginosa* IFO 3080 cells when the suspensions prepared from the same bacterial cells were incubated with these 3,4-dihydroxybenzoates. Both showed dose−response for this respiratory inhibition. These 3,4-dihydroxybenzoates also inhibited *P. aeruginosa* NADH oxidase by a membrane fraction prepared from the same bacterial cells, similar to those found for alkyl 3,4,5-trihydroxybenzoates (*41*). The IC_{50} value of 3.9 μM obtained for nonyl 3,4-dihydroxybenzoate is about 4-fold more potent than that of nonyl 3,4,5-trihydroxybenzoate. The possibility that the antifungal activity of alkyl 3,4-dihydroxybenzoates comes at least in part from their ability to inhibit respiratory systems cannot be entirely ruled out.

Antioxidant Activity. In most foods, the browning process has two components: enzymatic and nonenzymatic oxidation. This unfavorable darkening from oxidation generally results in a loss of nutritional as well as market value. The enzymatic oxidation can be prevented by tyrosinase inhibitors, and the nonenzymatic oxidation can be protected by antioxidants. As expected, one molecule of an alkyl 3,4-dihydroxybenzoate, regardless of the alkyl chain length, scavenges six molecules of DPPH. In a previous paper, 3,4,5-trihydroxybenzoic acid was reported to be a better antioxidant than 3,4-dihydroxybenzoic acid (*42*). This is consistent with our current result as illustrated in **Figure 7a**. However, their nonyl esters scavenged DPPH and the potency is almost the same as shown in **Figure 7b**. As long as DPPH scavenging activity is compared, alkyl 3,4-dihydroxybenzoates are as potent as alkyl 3,4,5-trihydroxylbenzoates. On the other hand, alkyl 3,4-dihydroxybenzoates were tested for their mushroom tyrosinase inhibitory activity. Although the resistance to the oxidation increases with increasing carbon chain length, alkyl 3,4-dihydroxybenzoates were eventually oxidized as substrates. Nonyl 3,4-dihydroxybenzoate was oxidized as a substrate at an extremely slow rate. The peak at 475 nm caused by dopachrome formation was not detected for several minutes. Since the enzyme activity was not observed exceeding 1 min, the inhibition seemingly follows Michaelis−Menten kinetics. However, this short observation is not practical to consider for food protection.

On the other hand, lipid peroxidation is known to be one of the major factors in deterioration during the storage and processing of foods, because it can lead to the development of unpleasant rancid or off flavors as well as potentially toxic end products (*43*). Hence, nonyl 3,4-dihydroxybenzoate's lipoxygenase inhibitory activity was also tested using soybean lipoxygenase-1. It was found to inhibit soybean lipoxygenese-1 with an IC_{50} of 0.25 μM competitively. Neither 3,4-dihydroxybenzoic acid nor nonanol alone inhibited soybean lipoxygenase-1 up to 200 μM. In connection with this, L-ascorbic acid (also

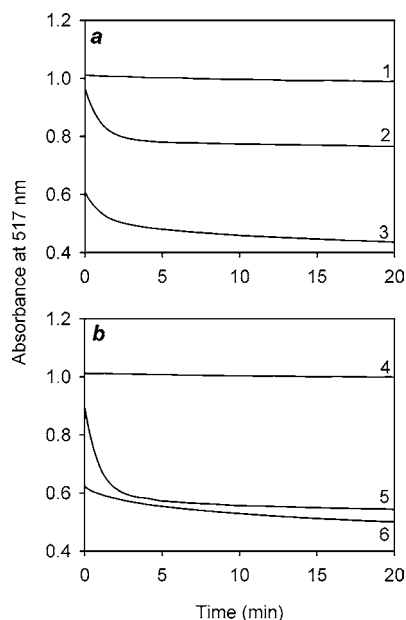**Figure 7.** Progress curves for the DPPH scavenging activity by 10 μM benzoic acids (**a**) or benzoates (**b**) for 20 min. Lines 1–3 represent the decay of absorbance at 517 nm by 3,5-dihydroxybenzoic, 3,4-dihydroxybenzoic, and gallic acid, respectively. Lines 4–6 represent the decay of absorbance at 517 nm by nonyl 3,5-dihydroxybenzoate, 3,4-dihydroxybenzoate, and gallate, respectively.

known as vitamin C) did not inhibit this enzyme up to 300 μM. Although intermediate free radicals are known to form during the catalytic cycle of lipoxygenases (*44*), they remain tightly bound at the active site, thus not being accessible for free radical scavengers. Hence, nonyl 3,4-dihydroxybenzoate inhibited soybean lipoxygenase-1 but is unlikely due to its capability of scavenging linoleic acid derived free radicals. In summary, nonyl 3,4-dihydroxybenzoate appears to combine both lipoxygenase inhibitory activity and free radical scavenging property in one agent and thus is an effective antioxidant.

In addition, 3,4-dihydroxybenzoic acid and its esters are known to chelate transition metal ions which are powerful promoters of free radical damage in both the human body (*8*) and foods (*9*). The chelation ability, rendering the metal ions inactive to participate in free radical generating reactions, should be of considerable advantage as an antioxidant. For example, alkyl 3,4-dihydroxybenzoates and 3,4-dihydroxybenzoic acid may suppress the superoxide-driven Fenton reaction, which is currently believed to be the most important route to active oxygen species (*45*). Gallic acid is known to inhibit iron absorption from diet, whereas the phenolic catechol groups seem to be of minor importance (*46*).

Conclusion. The primary fungicidal activity of amphipathic alkyl 3,4-dihydroxybenzoates likely comes from their ability to act as nonionic surfactants, similar to results found for alkyl gallates (*2*). However, it cannot be inferred that membrane damage is the only cause of the lethal effect. The alkyl 3,4-dihydroxybenzoates may enter into the cells through pores derived from membrane damage. It is also conceivable that some permeate in part by passive diffusion across the plasma membrane. Once inside the cells, alkyl 3,4-dihydroxybenzoates may inhibit various intercellular components. For example, nonyl 3,4-dihydroxybenzoate was found to inhibit the succinate-supported respiration of intact mitochondria isolated from rat liver (*47*). It is evident that alkyl 3,4-dihydroxybenzoates do not act by a single defined process but have multiple functions.
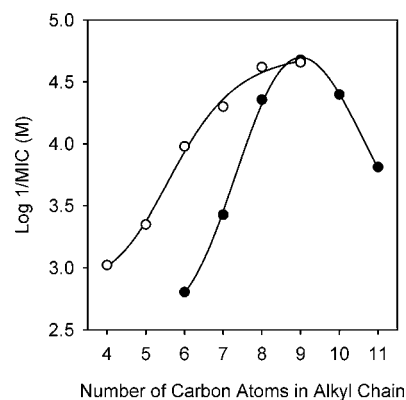

Figure 8. Comparison of antifungal activity of alkyl gallate (●) and alkyl 3,4-dihydroxybenzoate (○). The best curve fit was obtained with the peak-style equation ($y = 2.6 + 2.1 \exp(-0.5(\ln(x/9.0)/0.2))$, $R^2 = 0.9989$) by alkyl gallate or the sigmoidal equation ($y = 2.9 + 1.9/(1 + (x/5.9)^{-7.0})$, $R^2 = 0.9963$) by alkyl 3,4-dihydroxybenzoate.

However, the increased lipophilicity of alkyl 3,4-dihydroxybenzoates may affect their movement across the membrane (*48*) rather than their disruption of intercellular components and hence amphipathic alkyl 3,4-dihydroxybenzoates and 3,5-dihydroxybenzoates may act predominantly as surfactants against yeasts. The potency of the antifungal activity (MIC) of alkyl 3,4-dihydroxybenzoates against *S. cerevisiae* was increased with each additional CH$_2$ group. This is similar to results found for alkanols (*19*) and somewhat differs from data for alkyl 3,4,5-trihydroxybenzoates (*2*). In the latter case, the antifungal activity (MIC) against *S. cerevisiae* was a peak-style function of their lipophilicity as illustrated in **Figure 8**. *S. cerevisiae* was centered as the target organism in this paper, but alkyl 3,4-dihydroxybenzoates possess broad antimicrobial activity, similar to that found for alkyl 3,4,5-trihydroxybenzoates (*3*). For example, octyl 3,4-dihydroxybenzoate exhibits a broad antimicrobial spectrum similar to octyl 3,4,5-trihydroxybenzoate (*41*).

Safety is a primary consideration for antimicrobial agents, especially for those in food products. After alkyl 3,4-dihydroxybenzoates are consumed together with the food to which they are added as additives, these esters are hydrolyzed to 3,4-dihydroxybenzoic acid and the corresponding alcohols. Both are common plant components. In addition, the freed 3,4-dihydroxybenzoic acid acts as an antioxidant (*49, 50*); for instance, it has recently been reported to inhibit lipid peroxidation and to scavenge hydrogen peroxide (*51*) and superoxide anion generated enzymatically and nonenzymatically, even more potent than its ester form. The results obtained may provide a hint to the design of more appropriate multifunctional antimicrobial agents. However, there are contradictory reports regarding 3,4-dihydroxybenzoic acid. For example, 3,4-dihydroxybenzoic acid was previously reported to interact with Fe^{2+} and inhibit iron-induced oxidative DNA damage (*52*). Paradoxically, the same 3,4-dihydroxybenzoic acid was also described to act as a prooxidant causing copper-dependent DNA damage (*53*). In either case, the formation of *o*-quinone is a key biochemical pathway (*54*). The conflicting sequences of antioxidant potential in the different systems were also previously described (*55*). In biological systems, the specific reaction observed for in vitro incubations need not necessarily reflect the in vivo antioxidant behavior of a chemical. In addition to intrinsic chemical behavior, a further evaluation is needed not only from one aspect but also from a whole and dynamic perspective.

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