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Non-antibiotic Antibacterial Activity of Dodecyl Gallate

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In honor of Professor Andrew S. Kende's seventieth birthday.

Abstract—Dodecyl (C_{12}) gallate (3,4,5-trihydroxybenzoate) (**1**) was found to possess antibacterial activity specifically against Gram-positive bacteria, in addition to its potent antioxidant activity. The time–kill curve study indicates that this amphipathic gallate exhibits bactericidal activity against methicillin resistant *Staphylococcus aureus* (MRSA) strains. Dodecyl (lauryl) gallate inhibited oxygen consumption in whole cells and oxidation of NADH in membrane preparation. The antibacterial activity of this gallate comes in part from its ability to inhibit the membrane respiratory chain. As far as alkyl gallates are concerned, their antimicrobial spectra and potency depend in part on the hydrophobic portion of the molecule.

Introduction

In our continuing search for antimicrobial agents as food preservatives, propyl (C_3) (**2**), octyl (C_8) (**3**) and dodecyl (C_{12}) (**1**) gallates were previously tested for their antifungal activity against *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, *Candida albicans*, and *Aspergillus niger*.[1] Among the three gallates tested, only octyl gallate was effective against the fungi, whereas neither propyl nor dodecyl gallates showed any antifungal activity. The length of the alkyl group was found to be associated with the activity to a large extent. This conclusion can be supported by the fact that antifungal activity begins to level off, and a point is soon reached beyond which antifungal activity disappears (the so-called 'cutoff' phenomenon) and the length of the dodecyl group is beyond this point. It is well established that the hydrophobicity of molecules is often related to biological action;[2] however, the rationale for this, especially the role of the hydrophobic portion, is still poorly understood and widely debated. Alkyl gallates are a superior model for structure–antibacterial activity relationship (SAR) study because these molecules are common to possess the same hydrophilic portion, the pyrogallol moiety. Thus, providing a better understanding of the role of the hydrophobic alkyl portion. Accumulation of this knowledge may provide a more rational and scientific approach to design safe and effective antimicrobial agents.

During the current experiment, we became aware that dodecyl gallate exhibited antibacterial activity specifically against Gram-positive bacteria.[1] Since this gallate is one of the three gallates which are currently permitted for use as antioxidant additives in food and cosmetic products,[3] its mode of selective antibacterial action on a molecular basis as well as the role of hydrophobic alkyl moiety has been studied. The work has been communicated in part[1] and is described in full.

Results

Dodecyl gallate (**1**) (see Fig. 1 for structures) was tested for its antimicrobial activity against the 16 selected microorganisms and compared with those of propyl and octyl gallates. The results are listed in Table 1. Dodecyl gallate inhibited the growth of all the Gram-positive bacteria tested, *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus mutans*, *Micrococcus luteus*, *Propionibacterium acnes*, and *Brevibacterium ammoniagenes*. Among them, *P. acnes* was found to be the most susceptible to dodecyl gallate with an MBC of 6.25 μg/mL (18 μM), while *S. mutans* was the least with an MBC of 100 μg/mL (296 μM). Subsequently, the MBCs against *S. aureus*, *B. subtilis*, *M. luteus*, and *B. ammoniagenes* were 25 μg/mL (74 μM). The potency of dodecyl gallate against the bacteria tested is slightly more potent than

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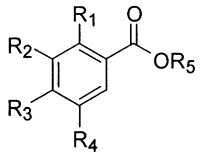
that of octyl gallate (**3**), but propyl gallate (**2**) did not show any notable activity. The differences in MICs and MBCs of octyl and dodecyl gallates against Gram-positive bacteria were not more than 2-fold, suggesting that residual bacteriostatic activity is unlikely involved. Since gallic acid did not exhibit any bactericidal activity against this MRSA strain up to 1600 μg/mL, the alkyl group must play a role in eliciting the activity.



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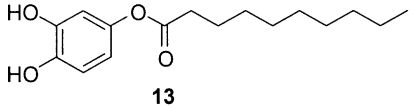
4: $R_1 = R_2 = R_3 = R_4 = H, R_5 = (CH_2)_{11}CH_3$
5: $R_1 = OH, R_2 = R_3 = R_4 = H, R_5 = (CH_2)_{11}CH_3$
6: $R_1 = R_3 = R_4 = H, R_2 = OH, R_5 = (CH_2)_{11}CH_3$
7: $R_1 = R_2 = R_4 = H, R_3 = OH, R_5 = (CH_2)_{11}CH_3$
8: $R_1 = R_4 = H, R_2 = R_3 = OH, R_5 = (CH_2)_{11}CH_3$
9: $R_1 = R_3 = H, R_2 = R_4 = OH, R_5 = (CH_2)_{11}CH_3$
10: $R_1 = R_2 = OH, R_3 = R_4 = H, R_5 = (CH_2)_{11}CH_3$
11: $R_1 = R_4 = H, R_2 = OH, R_3 = OCH_3, R_5 = (CH_2)_{11}CH_3$
12: $R_1 = R_4 = H, R_2 = OCH_3, R_3 = OH, R_5 = (CH_2)_{11}CH_3$



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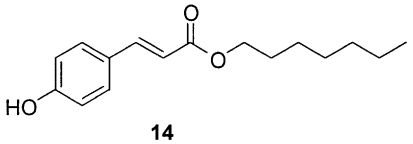

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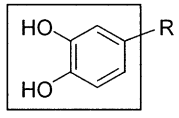

a

Figure 1. Chemical structures of gallates and related compounds.

In the case against *S. aureus*, the comparable MBCs were found against six *S. aureus* strains, including two methicillin resistant and two penicillin resistant strains. The current study was targeted against *S. aureus* (MRSA) ATCC 33591 strain as an example unless otherwise specified. The potency of dodecyl gallate against *S. aureus* strains is slightly more potent than that of octyl gallate as listed in Table 2. It is worthwhile to add that one of the most commonly occurring food poisonings is caused by the ingestion of the enterotoxin formed in food during growth of certain strains of *S. aureus*.

The bactericidal effect of dodecyl gallate against *S. aureus* (MRSA) was confirmed by the time–kill curve experiment as shown in Figure 2. Cultures of this MRSA strain, with a cell density of 5.0×10^5 CFU/mL, were exposed to three different concentrations of dodecyl gallate. The number of viable cells was determined following different periods of incubation with dodecyl gallate. The final cell count at MBC of dodecyl gallate was $1/10^4$ of the control, indicating that dodecyl gallate at MBC was not bactericidal. Complete lethality occurred at 2 × MBC. No viable cells were detected after being exposed to 50 μg/mL (2 × MBC) of dodecyl gallate during cultivation. The result indicates that the amount of the drug's molecules needed to be increased by increasing the number of viable cells. It seems that dodecyl gallate is unlikely to disrupt specific target proteins such as cell-surface receptors or signal transduction proteins.

Subsequently, the effects of dodecyl gallate against *S. aureus* were further tested while holding viable cell number in the presence of chloramphenicol, which is known to restrict cell division by inhibiting bacterial protein synthesis. When the cells were treated with

Table 1. Antimicrobial activity of propyl (C_3), octyl (C_8) and dodecyl (C_{12}) gallates

Microorganisms tested	MIC (*MBC* or *MFC*) (μg/mL)		
	C_3	C_8	C_{12}
Bacillus subtilis	800 (*1600*)	12.5 (*25*)	25 (*50*)
Brevibacterium ammoniagenes	1600 (*3200*)	25 (*50*)	12.5 (*25*)
Micrococcus luteus	1600 (*3200*)	12.5 (*25*)	12.5 (*25*)
Streptococcus mutans	400 (*800*)	50 (*50*)	100 (*100*)
Staphylococcus aureus	1600 (*3200*)	25 (*50*)	12.5 (*25*)
S. aureus (MRSA)	1600 (*3200*)	25 (*50*)	12.5 (*25*)
Propionibacterium acnes	800 (*800*)	25 (*25*)	6.25 (*6.25*)
Escherichia coli	1600 (*1600*)	> 800 (> *800*)	> 800 (> *800*)
Pseudomonas aeruginosa	3200 (> *3200*)	> 800 (> *800*)	> 800 (> *800*)
Enterobacter aerogenes	3200 (> *3200*)	> 800 (> *800*)	> 800 (> *800*)
Proteus vulgaris	400 (*400*)	25 (*50*)	> 800 (> *800*)
Salmonella choleraesuis	1600 (> *3200*)	12.5 (*12.5*)	25 (*50*)
Saccharomyces cerevisiae	3200 (> *3200*)	25 (*25*)	> 1600 (> *1600*)
Zygosaccharomyces bailii	> 3200 (> *3200*)	50 (*50*)	> 1600 (> *1600*)
Candida albicans	3200 (> *3200*)	25 (*25*)	> 400 (> *400*)
Aspergillus niger	> 3200 (> *3200*)	50 (*100*)	> 400 (> *400*)

Numbers in *italic* type in parentheses are *MBC* or *MFC*.

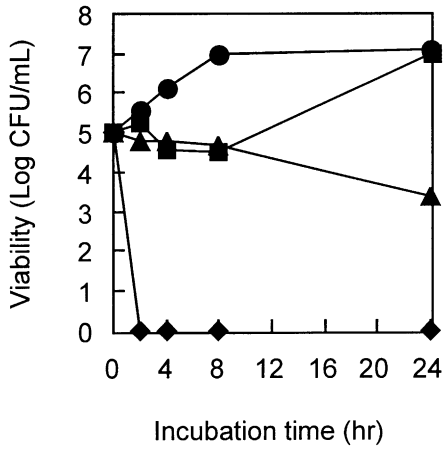

Figure 2. Bactericidal effects of dodecyl gallate against *S. aureus* ATCC 33591. Exponentially growing cells of *S. aureus* were inoculated into NYG broth and then cultured at 37 °C without shaking. Dodecyl gallate: 0 (●), 12.5 (■), 25 (▲), 50 (◆) μg/mL.

chloramphenicol prior to the addition of dodecyl gallate to the culture, the bactericidal effect of dodecyl gallate was reduced within 4 h after addition of the gallate, as shown in Figure 3. However, even in this case, bactericidal effect of dodecyl gallate in the chloramphenicol-pretreated cells was expressed in the same as those untreated after 24 h cultivation. Dodecyl gallate rapidly killed the exponentially growing cells of *S. aureus*. In addition, decrease in viability of the chloramphenicol-treated cells was also observed. However, the rate of decrease of cell number in the chloramphenicol-treated cells was slower than that in the exponentially growing cells. This observation excludes several modes of action for dodecyl gallate such as inhibition of DNA, RNA and protein, and cell wall synthesis in vivo.

Dodecyl gallate was also noted to exhibit bactericidal activity against *B. subtilis*. The result observed differs from those of aliphatic alkanols[4] and (2*E*)-alkenals,[5] which did not show any bactericidal activity against this spore-forming bacterium up to 800 μg/mL. This indicates that dodecyl gallate inactivates spores of *B. subtilis* as a sporicide.[6] The spore-forming species of the genera *Bacillus* is difficult to control, because endospores (i) are formed at an intracellular site, (ii) are very retractile, and (iii) are resistant to heat, ultraviolet light, and desiccation. Hence, the sporicidal activity of dodecyl gallate would appear to be of great overall value.

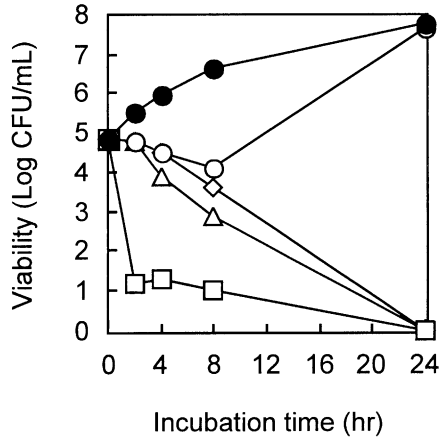

Figure 3. Effect of dodecyl gallate on the chloramphenicol-treated cells of *S. aureus* ATCC 33591. The cells of *S. aureus* were incubated with (○, □, △, ◇) or without (●) 12.5 μg/mL of chloramphenicol in NYG broth at 37 °C. Dodecyl gallate (50 μg/mL) was added at 0 (□), 2 (△), 4 (◇) h.

In general, gallates are not effective against the Gram-negative bacteria selected, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, and *Proteus vulgaris*. No gallate has been found to exhibit antibacterial activity against *P. aeruginosa* and *E. aerogenes*. As expected, neither octyl nor dodecyl gallates exhibited any antibacterial activity against these Gram-negative bacteria up to 1600 μg/mL. However, dodecyl gallate was noted to inhibit the growth of *Salmonella choleraesuis* with an MBC of 50 μg/mL (150 μM). No differences in MIC and MBC are noted, suggesting that its activity is bactericidal. This food borne bacterium is the only Gram-negative bacterium susceptible to alkyl gallates.

Dodecyl gallate can be considered as head and tail structures similar to aliphatic alkanols,[4] and hence the mode of antibacterial action is expected to act as a surface-active agent (surfactant). However, the results obtained do not support the possibility that dodecyl gallate acts as a surfactant, at least not as a major contributor. If this is so, alternative functions may need to be considered. Notably, dodecyl gallate inhibited the oxygen consumption of *P. aeruginosa* IFO 3080 cells when the suspensions prepared from the same bacterial cells were incubated with dodecyl gallate, as shown in Figure 4. Dodecyl gallate showed dose–response for this respiratory inhibition. The same gallate also inhibited *P. aeruginosa* NADH oxidase by a membrane fraction prepared from the same bacterial cells as shown in Figure 5. The results observed indicate that dodecyl gallate inhibits the bacterial membrane respiratory chain. It should be noted that *P. aeruginosa* IFO 3080 strain was used as previously described.[7] In connection with this,

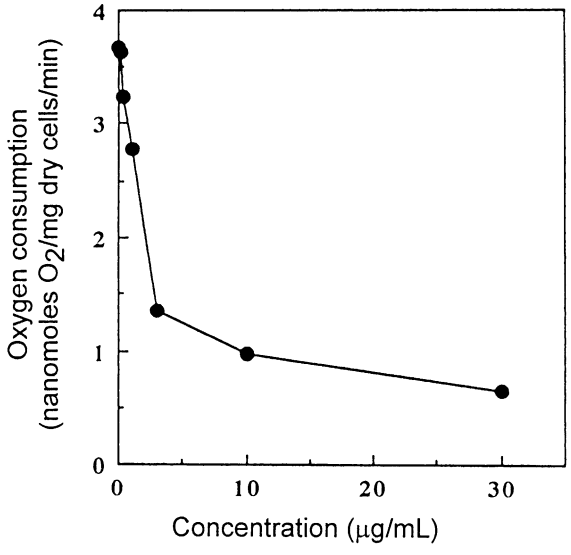

Figure 4. Effect of dodecyl gallate on respiratory activity in *P. aeruginosa* IFO 3080 cells. Each plot is the mean of triplicate determinations.

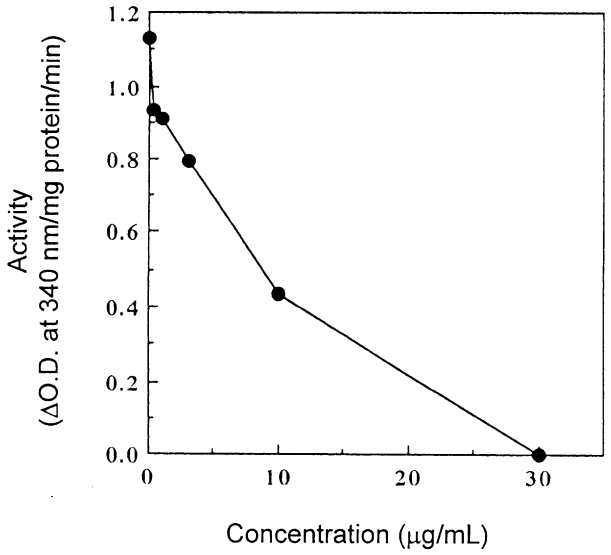

Figure 5. Effect of dodecyl gallate on NADH oxidase of a membrane fraction isolated from *P. aeruginosa* IFO 3080. Each plot is the mean of triplicate determinations.

dodecyl gallate inhibited the growth of *P. aeruginosa* IFO 3080 strain with an MIC of 12.5 μg/mL but not ATCC 10145 strain up to 800 μg/mL. This IFO 3080 strain is not susceptible to most antibiotics from microbial origin but is sensitive to some antibacterial phytochemicals.[8,9] In addition, dodecyl gallate also inhibited the oxygen consumption of *M. luteus* ATCC 4698 cells when the suspensions prepared from the same bacterial cells were incubated with dodecyl gallate. This inhibition by dodecyl gallate showed dose–response and the concentration found to inhibit oxygen consumption is approximately comparable to that causing bactericidal activity against *M. luteus*.

The 'hydrolyzable' ester group was selected in order to avoid undesired side effects, particularly endocrine disrupting activity of environmentally persistent estrogen mimics[10] such as alkylphenolic compounds.[11] The ester group did not exist in the alkanol structure and may be related to elicit the activity. Alkanols themselves are known to exhibit the antibacterial activity,[4] so the possibility of bacteria exuding an esterase that hydrolyzes dodecyl gallate to gallic acid and dodecanol was first taken into account. However, this possibility can be ruled out since dodecyl benzoate (**4**), dodecyl 2-hydroxybenzoate (**5**) and dodecyl 3-hydroxybenzoate (**6**) did not exhibit any antibacterial activity against *S. aureus* up to 200 μg/mL, as shown in Table 2. It should be noted however that dodecyl 4-hydroxybenzoate (**7**) exhibited some bactericidal activity against Gram-positive bacteria.

The surfactant concept was derived in an additional experiment that indicates antifungal octyl and nonyl gallates rapidly adsorbed onto the surface of *S. cerevisiae* cells but dodecyl gallate did not, as shown in Figure 6. It appears that *S. cerevisiae* showed different affinities to gallates having different alkyl chain length. The adsorbing sites may not be specific but need to be clarified in order to explain the difference observed. On the other hand, most of the dodecyl gallate did not adsorb onto the cell surface of *S. cerevisiae* and remained in the water based medium, probably in the form of insoluble monolayer or spread film.[12] This may reveal in part why dodecyl gallate did not show any effects on eukaryotic microorganisms such as *S. cerevisiae*.

Propyl, octyl and dodecyl gallates are currently permitted for use as antioxidant additives in food. Antioxidants usually protect cells as radical scavengers. For example, gallic acid and its esters reduced cell damage induced by hydroxy radicals and hydrogen peroxides in bacteria, *Salmonella typhimurium* and *E. coli*.[13] Paradoxically, these gallates have been reported to trigger apoptotic pathway in several cell lines accompanied by reactive oxygen species (ROS) generation. Gallates were reported to induce apoptosis in human leukemia HL60 RG and to show cytotoxic effects on other cell lines.[14–17] In these apoptotic processes, the generation of ROS is thought to contribute to the initiation of apoptosis.[15,18,19] *p*-Nonylphenol, octyl and dodecyl gallates were found to induce cellular ROS generation *p*-Nonylphenol as shown in Figure 7. Membrane lipids are abundant in unsaturated fatty acids. The oxidation of these unsaturated fatty acids leads to a decrease in the membrane fluidity and disruption of membrane structure and function. Hence, ROS generation may explain their bactericidal action. However, ROS generation in *S. aureus* cells caused by dodecyl gallate is not directly associated with the bactericidal action since the antioxidants such as α-tocopherol, L-ascorbate, and *N*-acetylcysteine did not exhibit any protective effect. Rather, octyl and dodecyl gallates act as antioxidants and protect from oxidative damage.

Interestingly, dodecyl 3,4-dihydroxybenzoate (**8**) also exhibited antibacterial activity against *S. aureus* whereas neither dodecyl 3,5-dihydroxybenzoate (**9**) nor dodecyl 2,3-dihydroxybenzoate (**10**) exhibited any activity. The results indicate that the catechol moiety of '***a***' is prerequisite to elicit the activity. This postulate can be supported by the observation that neither dodecyl 3-hydroxy-4-methoxybenzoate (**11**) nor nonyl 4-hydroxy-3-methoxybenzoate (**12**) shows any antibacterial activity. The potency of dodecyl 3,4-dihydroxybenzoate is nearly comparable with that of dodecyl 3,4,5-trihydroxybenzoate (gallate), indicating that the additional hydroxyl group to ***a*** is superfluous. On the other hand, dodecyl 3,5-dihydroxybenzoate was noted to be effective against *B. ammoniagenes* with an MBC of 100 μg/mL. *B. ammoniagenes* is the only susceptible bacterium to dodecyl 3,5-dihydroxybenzoate. The reason for this is unknown. In connection with the aforementioned ester group, this group is not directly related to the antimicrobial activity since both 3,4-dihydroxyphenyl decanoate (**13**) and heptyl coumarate (heptyl 4-hydroxycinnamate) (**14**) showed similar antibacterial activity. Dodecyl gallate exhibited antibacterial activity and scavenging activity on 1,1-diphenyl-2-*p*-picrylhydrazyl (DPPH) radicals, indicating that pyrogallol moiety is associated with the antibacterial and scavenging activity. In connection, one molecule of alkyl gallate, regardless of the alkyl chain length, scavenges six molecules of DPPH.

Discussion

Mode of antibacterial action of octyl gallate seems to differ from its antifungal action. In the case against *S. cerevisiae*, the primary antifungal activity of octyl gallate was described to come from its ability to act as a nonionic surfactant.[1] On the basis of the data obtained, it appears that the catechol moiety of '***a***' seems to be essential to elicit the antifungal and antibacterial activity, and that the length of the alkyl group is also related to the activity. The hydrophilic catechol moiety binds with an intermolecular hydrogen bond like a 'hook' in attaching itself to the hydrophilic head portion of the membrane, and then the hydrophobic tail portion of the molecule is able to enter into the membrane lipid bilayer portion. This creates, as a result, disorder in the fluid bilayer of the membrane. The antibacterial activity of dodecyl gallate is unlikely due to this surfactant action, at least not as a major contributor. The data obtained so far suggest that biochemical mechanisms play a more essential role in antibacterial activity of alkyl gallates.

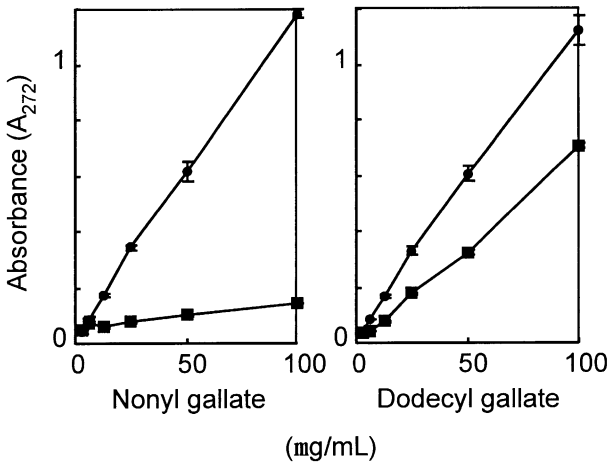

Figure 6. Adsorption of nonyl and dodecyl gallates to the cells of *S. cerevisiae* ATCC 7754. After each gallate was mixed with (■) or without (●) yeast cells (10^8 cells/mL), the suspension was vortexed for 5 s. Absorbance of the supernatant obtained by centrifugation for 2 min was measured.

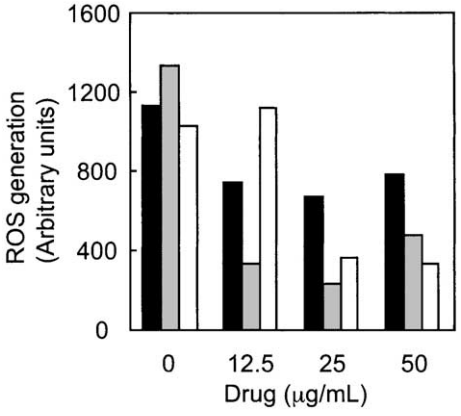

Figure 7. Effect of *p*-nonylphenol, and octyl and dodecyl gallates, on ROS generation in *S. aureus* ATCC 33591 cells. After the cells were incubated with *p*-nonylphenol (black bar), octyl gallate (gray bar), and dodecyl gallate (white bar) in NYG medium at 37 °C for 60 min, the amount of ROS generated was measured.

The hydroxybenzoates may enter in part into the cells through pores derived from membrane damage. However, the increased lipophilicity of gallates should affect in general their movement more into the membrane lipid bilayer portions.[20] On the basis of the data obtained, it may not be illogical to assume that highly lipophilic dodecyl gallate no longer maintains the balance of the head and tail structure against *S. cerevisiae*, so most likely does not act as a surfactant. It seems that most of the dodecyl gallate exists in the form of insoluble monolayer or spread film in the water based test medium.[12] Even if the part of the molecules dissolved in the medium may not pass across the cytoplasmic membrane it may diffuse into the lipid bilayer portions. Then, pyrogallol moiety needs to be taken into account. In addition, the length of the alkyl chain plays a role in eliciting the activity since gallic acid did not show any activity up to 1600 μg/mL. Hence, the actual solubility of the test compounds in the water based test media seems to be an important factor.

The difference between antifungal and antibacterial actions of gallates needs to be taken into account. Prokaryotic and eukaryotic microorganisms are known to differ in many ways. For example, the electron transport chain (ETC) involved in the respiratory chain is located in the cytoplasmic membrane in bacteria, while in fungi it is located in the mitochondria. In the current study, this difference must be taken into account because the process by which alkyl gallates reach the action sites in

Table 2. Antibacterial activity of dodecyl benzoates against Gram-positive bacteria

Compounds tested	MIC (MBC) (μg/mL)			
	B. subtilis	*S. aureus*	*M. luteus*	*B. ammoniagenes*
Dodecyl Benzoate	>200 (>*200*)	>200 (>*200*)	>200 (>*200*)	>200 (>*200*)
Hydroxybenzoate	>400 (>*400*)	100 (*100*)	25 (*25*)	25 (*50*)
3,5-Dihydroxybenzoate	>200 (>*200*)	>200 (>*200*)	200 (>*200*)	100 (*100*)
3,4-Dihydroxybenzoate	50 (*50*)	12.5 (*50*)	6.25 (12.5)	12.5 (*25*)
3,4,5-Trihydroxybenzoate	25 (*50*)	12.5 (*25*)	12.5 (*25*)	12.5 (*25*)
Methicillin	1.56 (*6.25*)	1.56 (>*6.25*)	6.25 (*6.25*)	25 (*25*)

Numbers in *italic* type in parentheses are *MBC*.

living microorganisms is usually neglected in the cell-free experiment. The inner and outer surfaces of the membrane are hydrophilic while the interior is hydrophobic. It is logical to assume that most of the lipophilic dodecyl gallate molecules being dissolved in the medium are incorporated into the lipid bilayers[20] without perturbing the lipid.[21] Once inside the membrane lipid bilayers, this gallate may inhibit the ETC, perhaps by interfering with the redox reactions. The pyrogallol moiety apparently plays a major role for this interference. In the case against fungi, dodecyl gallate unlikely enters into the cells and cannot reach the mitochondria. However, the possibility that dodecyl gallate enters in part into the cells through pores derived from its membrane damage cannot be entirely ruled out. If this is the case, the molecules entered into the cells may be dissolved into the mitochondrial outer membrane and rarely reach the inner membrane where the ETC is located. This may reveal in part why dodecyl gallate did not show any effects on eukaryotic microorganisms such as *S. cerevisiae* in which the respiration depends on a mitochondrial ETC. The length of the alkyl chain apparently regulates the different susceptibility between octyl and dodecyl gallates. As a result, dodecyl gallate showed only antibacterial activity specifically against Gram-positive bacteria whereas octyl gallate exhibited a broad antimicrobial spectrum including antifungal and antibacterial activity.[1]

In the time–kill experiment against *S. aureus*, dodecyl gallate rapidly reduced the number of viable cells within the first 1 h after adding this gallate and then slowed thereafter, suggesting that different modes of action might be involved. For example, it may act largely as a surfactant within the first 1 h and then as a respiratory inhibitor thereafter. The data obtained are consistent with an effect on the bulk membrane rather than a direct interaction of the specific target proteins. In addition, dodecyl gallate does not act by a single defined process but rather has multiple functions such as a nonionic surfactant and respiratory inhibitor by which it exerts its bactericidal action. In contrast to dodecyl gallate, octyl gallate first may act as a surfactant and then enter into the cytoplasm through pores derived from membrane.

Dodecyl gallate acts in general as a multifunctional agent in food—at least as an antimicrobial, antioxidant and tyrosinase inhibitory agent.[22] After dodecyl gallate is consumed together with the food to which it is added as an additive, this ester is hydrolyzed to the original gallic acid and dodecanol. The former still acts as a potent antioxidant, and both are common plant components. More specifically, freed gallic acid acts as antioxidant; for instance it scavenges superoxide anion generated enzymatically and non-enzymatically—even more potent than its ester forms. Gallic acid is known in many plants such as blackberry bark, henna, tea, mango and uva ursi. Dodecanol is reported in a large number of essential oils. As aforementioned, the three gallates—propyl, octyl and dodecyl—are currently permitted for use as antioxidant additives in food; therefore, the antibacterial activity noticed is their additional advantage.

Experimental

Chemicals

Propyl, octyl, and dodecyl gallates used for the assay were purchased from Aldrich Chemical Co. (Milwaukee, WI). Dodecyl gallate was recrystallized prior to the use. Methicillin, chloramphenicol, and DPPH were obtained from Sigma Chemical Co. (St. Louis, MO). For the experiment, all compounds were first dissolved in *N,N*-dimethylformamide (DMF) which was purchased from EM Science (Gibbstown, NJ). The concentration of DMF in each medium was always 1%.

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 dilute aqueous citric acid solution, saturated aqueous $NaHCO_3$ solution and water, dried over $MgSO_4$, and evaporated. The crude products were purified by chromatography (SiO_2; elution with $CHCl_3$–MeOH, 98:2). Structures of the synthesized esters were established by spectroscopic methods (IR, MS, and NMR). Their analogues such as heptyl coumarate and 3,4-dihydroxyphenyl decanoate were also synthesized in the same manner.

Dodecyl benzoate (4). This was obtained in 82% yield as a colorless solid: ^{1}H NMR (270 MHz, $CDCl_3$, δ): 0.88 (t, J = 6.6 Hz, 3H, –CH_3), 4.20 (t, J = 6.6 Hz, 2H, –OCH_2), 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, 1652, 1560, 1480, 1395, 1250, 1175 cm^{-1}.

Dodecyl 3-hydroxybenzoate (6). This was obtained in 68% yield as a colorless solid: ^{1}H NMR (270 MHz, $CDCl_3$, δ): 0.82 (t, J = 6.6 Hz, 3H, –CH_3), 3.23 (m, 2H, –CH_2), 4.24 (t, J = 7.0 Hz, 2H, –OCH_2), 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}.

Dodecyl 4-hydroxybenzoate (7). This was obtained in 95% yield as a colorless solid: ^{1}H NMR (270 MHz, $CDCl_3$, δ): 0.88 (t, J = 6.6 Hz, 3H, –CH_3), 3.66 (t, J = 6.6 Hz, 2H, –CH_2), 4.26 (t, J = 6.6 Hz, 2H, –OCH_2), 6.85 (d, J = 8.7 Hz, 2H, ArH), 7.92 (d, J = 8.7 Hz, 2H, ArH); IR (KBr) 3489, 3165, 2954, 2922, 1714, 1695, 1611, 1470, 1280, 1164 cm^{-1}.

Dodecyl 2-hydroxybenzoate (5). This was obtained in 63% yield as a colorless solid: ^{1}H NMR (270 MHz, $CDCl_3$, δ): 0.88 (t, J = 6.6 Hz, 3H, –CH_3), 3.79 (m, 2H, –CH_2), 4.87 (t, J = 6.8 Hz, 2H, –OCH_2), 7.05 (d, J = 7.8 Hz, 1H, ArH), 7.18 (t, J = 7.8 Hz, 1H, ArH), 7.52 (t, J = 7.8 Hz, 1H, ArH), 7.98 (d, J = 7.8 Hz, 1H, ArH);

IR (KBr) 3360, 2960, 2890, 1640, 1590, 1552, 1480, 1395, 1250, 1170 cm^{-1}.

Dodecyl protocatechuate (dodecyl 3,4-dihydroxybenzoate) (8). This was obtained in 81% yield as a colorless solid: ^1H NMR (270 MHz, $CDCl_3$, δ): 0.89 (t, $J = 6.6$ Hz, 3H, $–CH_3$), 4.24 (t, $J = 6.6$ Hz, 2H, $–OCH_2$), 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) 3530, 3360, 2960, 2890, 1700, 1625, 1550, 1480, 1395, 1290, 1180 cm^{-1}.

Dodecyl 3,5-dihydroxybenzoate (9). This was obtained in 74% yield as a colorless solid: ^1H NMR (270 MHz, $CDCl_3$, δ): 0.88 (t, $J = 6.6$ Hz, 3H, $–CH_3$), 4.21 (t, $J = 6.6$ Hz, 2H, $–OCH_2$), 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 $C_{19}H_{30}O_4$, 322.2140; found 322.2144.

Dodecyl 2,3-dihydroxybenzoate (10). This was obtained in 74% yield as a colorless solid: ^1H NMR (270 MHz, $CDCl_3$, δ): 0.88 (m, 3H, $–CH_3$), 3.86 (m, 2H, $–CH_2$), 4.92 (t, $J = 6.8$ Hz, 1H, $–OCH_2$), 6.79 (d, $J = 7.8$ Hz, 1H, ArH), 7.03 (t, $J = 7.8$ Hz, 1H, ArH), 7.53 (d, $J = 7.8$ Hz, 1H, ArH); IR (KBr) 3100, 2960, 2860, 1716, 1660, 1545, 1480, 1395, 1272 cm^{-1}.

Dodecyl 3-hydroxy-4-methoxybenzoate (11). This was obtained in 84% yield as a colorless solid: ^1H NMR (270 MHz, $CDCl_3$, δ): 0.88 (t, $J = 6.6$ Hz, 3H, $–CH_3$), 4.20 (s, 3H, $–OCH_3$), 4.26 (t, $J = 6.6$ Hz, 2H, $–OCH_2$), 6.73 (d, $J = 8.7$ Hz, 1H, ArH), 7.43 (dd, $J = 2.2$, 8.7 Hz, 1H, ArH), 7.65 (d, $J = 2.2$ Hz, 1H, ArH); IR (KBr) 3290, 2960, 2890, 1690, 1630, 1600, 1480, 1395, 1251, 1180 cm^{-1}.

Dodecyl 4-hydroxy-3-methoxybenzoate (12). This was obtained in 66% yield as a colorless solid: ^1H NMR (270 MHz, $CDCl_3$, δ): 0.88 (t, $J = 6.6$ Hz, 3H, $–CH_3$), 3.89 (s, 3H, $–OCH_3$), 4.20 (t, $J = 6.6$ Hz, 2H, $–OCH_2$), 6.78 (d, $J = 8.7$ Hz, 1H, ArH), 7.47 (dd, $J = 2.2$, 8.7 Hz, 1H, ArH), 7.53 (d, $J = 2.2$ Hz, 1H, ArH); IR (KBr) 3360, 2960, 2890, 1645, 1600, 1530, 1480, 1395, 1252, 1170 cm^{-1}.

3,4-Dihydroxyphenyl decanoate (13). This was obtained in 84% yield as a colorless solid: ^1H NMR (270 MHz, $CDCl_3$, δ): 0.89 (t, $J = 6.6$ Hz, 3H, $–CH_3$), 2.60 (t, $J = 6.8$ Hz, 2H, $–CH_2$), 6.18 (s, 1H), 6.31 (d, $J = 7.8$ Hz, 1H, ArH), 6.57 (d, $J = 7.8$ Hz, 1H, ArH). IR (nujol) 3480, 3300, 2780, 2720, 1700, 1750, 1650, 1620, 1540, 1250, 1190, 1160 cm^{-1}.

Heptyl coumarate [heptyl 3-(4-hydroxyphenyl)-2-propenoate] (14). Obtained in 71% yield as a colorless solid: ^1H NMR (270 MHz, $CDCl_3$, δ): 0.89 (t, $J = 6.6$ Hz, 3H, $–CH_3$), 4.18 (t, $J = 6.6$ Hz, 2H, $–OCH_2$), 6.25 (d, $J = 16.2$ Hz, 1H, –CH), 6.88 (d, $J = 7.8$ Hz, 1H, ArH), 7.0 (d, $J = 7.8$ Hz, 1H, ArH), 7.10 (s, 1H, ArOH), 7.57 (d, $J = 16.2$ Hz, 1H, –CH). IR (nujol) 3520, 3370, 2760, 2720, 1700, 1480, 1660, 1620, 1550, 1320, 1290, 1200 cm^{-1}.

Test strains

The microorganisms used for the assay, *B. subtilis* ATCC 9372, *B. ammoniagenes* ATCC 6872, *M. luteus* ATCC 4698, *S. mutans* ATCC 25175, *P. acnes* ATCC 11827, *S. aureus* ATCC 12598, *S. aureus* ATCC 33591 (MRSA), *S. aureus* ATCC 33592 (MRSA), *E. coli* ATCC 9637, *P. aeruginosa* ATCC 10145, *E. aerogenes* ATCC 13048, *P. vulgaris* ATCC 13315, *S. choleraesuis* ATCC 35640, *S. cerevisiae* ATCC 7754, *Z. bailii* ATCC 60483, *C. albicans* ATCC 18804, and *Aspergillus niger* ATCC 16404, used for this study were purchased from the American Type Culture Collection (Manassas, VA). *P. aeruginosa* IFO 3080 was available from our previous works.[7–9]

Media

The culture media for the bacteria consisted of 0.9% nutrient broth (BBL), 0.5% yeast extract (DIFCO), and 0.1% glucose (NYG) except for the case of *S. mutans*. For the culture of *S. mutans*, BHI consisting of 3.7% brain heart infusion (DIFCO), and for the culture of fungi, 2.5% malt extract (BBL) were used, respectively.

Antibacterial assay

Broth macrodilution methods were used as previously described[4,5] with slight modifications. Briefly, serial 2-fold dilutions of the test compounds were prepared in DMF, and 30 μL of each dilution was added to 3 mL of NYG broth. These were inoculated with 30 μL of an overnight culture of the test bacterium. After incubation of the cultures at 37 °C for 48°h, the minimum inhibitory concentration (MIC) was determined as the lowest concentration of the test compound that demonstrated no visible growth. The minimum bactericidal concentration (MBC) was determined as follows. After the determination of the MIC, 100-fold dilutions with drug-free NYG broth from each tube showing no turbidity were incubated at 37 °C for 48 h. The MBC was the lowest concentration of the test compound that was not visible in the drug-free cultivation.

Time–kill study

The cultivation with dodecyl gallate was performed the same as the above MIC assay. Samples were withdrawn at selected time points, and serial dilutions were performed in sterile saline before the samples were plated onto NYG agar plates. After the plates were incubated at 37 °C for 24 h, colony forming units (CFU) were estimated.

Adsorption test

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

Measurement of oxygen uptake

Exponentially growing *P. aeruginosa* or *S. aureus* cells were harvested and washed with saline by centrifugation. The cells were suspended in 50 mM phosphate buffer (pH 7.0) to give approximately 1 mg dry cells/mL. The test compound dissolved in DMSO was added to the reaction mixture and the O_2 consumption was measured polarographically at 30 °C with a YSI Model 43 Biological Oxygen Monitor.[7]

Preparation of bacterial cell membrane

Exponentially growing *P. aeruginosa* or *S. aureus* cells were harvested by centrifugation and then washed twice with distilled water. The cell paste was suspended in 50 mM Tris–HCl buffer (pH 7.4) containing 0.5 M sucrose and 20 mM $MgCl_2$, and then disrupted by ultrasound using a Sonifier 450 at 10 kc for 2 min at 4 °C. After centrifugation at 15,000*g* for 20 min, the supernatant was centrifuged at 105,000*g* for 90 min. The resultant precipitate was washed by centrifugation at 105,000*g* for 60 min with 10 mM Tris–HCl buffer (pH 7.4) containing 0.5 M sucrose and 10 mM $MgCl_2$. The precipitate was resuspended in the same buffer.[23]

Enzyme assay

NADH oxidase activity was assayed by measuring the decrease in the absorbancy at 340 nm. The reaction mixture contained 0.1 M Tris–HCl buffer (pH 7.5), 200 μM NADH and membrane fraction (equivalent to 2 mg protein).[24]

Measurement of ROS production

Cellular ROS production was examined by a method dependent on intracellular deacylation and oxidation of 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) to the fluorescent compound 2′,7′-dichlorofluorescein (DCF). This probe was highly reactive with hydrogen peroxide and has been used in evaluating intracellular ROS generation.[25] After preincubation of the *S. aureus* cells (10^8 cells/mL) in NYG medium with 40 μM DCFH-DA at 37 °C for 60 min, the cell suspensions (1.0 mL) were withdrawn and further treated with each chemical for the indicated time and then washed and resuspended in 100 μL of phosphate-buffered saline. Fluorescence intensity of the cell suspension (100 μL) containing 10^8 cells was read with a Cytoflow 2300 fluorescence spectrophotometer (Millipore Co.) with excitation at 480 nm and emission at 530 nm. The arbitrary units were based directly on fluorescence intensity.

DPPH radical scavenging assay

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 test tube. Then, 0.03 mL of the sample solution (dissolved in DMSO) was added to the tube and incubated at 25 °C for 20 min. The absorbance at 517 nm (DPPH, $\varepsilon = 8.32 \times 10^3$) was recorded. As control, 0.03 mL of DMSO was added to the tube. From decrease of the absorbance, scavenging activity was calculated and expressed as scavenged DPPH molecules per molecule.

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