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Molecular Design of Multifunctional Antibacterial Agents Against Methicillin Resistant *Staphylococcus aureus* (MRSA)

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Abstract—Antibacterial activity of a series of alkyl gallates (3,4,5-trihydroxybenzoates) against Gram-positive bacteria, especially methicillin resistant *Staphylococcus aureus* (MRSA) strains was evaluated. Gram-positive bacteria are all susceptible to alkyl gallates. Dodecyl gallate was the most effective against MRSA ATCC 33591 strain with the minimum bactericidal concentration (MBC) of 25 μg/mL (74 μM). The time-kill curve study showed that dodecyl gallate was bactericidal against this MRSA strain at any growth stage. This activity was observed even in the chloramphenicol-treated cells, but the rate of decrease of cell number was slower than that in the exponentially growing cells. The bactericidal activity of medium-chain alkyl gallates 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. Subsequently, the same series of alkyl protocatechuates (3,4-dihydroxybenzoates) were studied and the results obtained are similar to those found for alkyl gallates. The length of the alkyl chain is not a major contributor but is related to the activity.

Introduction

In our previous paper, we reported antifungal activity of a series of alkyl gallates against *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii* and *Candida albicans*. Their primary antifungal activity against these yeasts was described to come from their ability to act as nonionic surfactants,[1,2] similar to those found for medium-chain alkanols.[3] The maximum antimicrobial activity depends on the hydrophobic alkyl (tail) chain length from the hydrophilic pyrogallol moiety (head). Namely, the hydrophilic head part binds with an intermolecular hydrogen bond like a 'hook' attaching itself to hydrophilic portion of the membrane, and then 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.[4] During this study we became aware that the same alkyl gallates were also effective against Gram-positive bacteria.

Due to the adaptability, *Staphylococcus aureus* can easily develop resistance to commonly used antibiotics. This resistance involves the enzymatic inactivation and is often transferred to other bacteria by a variety of gene transfer mechanisms.[5] Hence, there is a great need for effective antibacterial agents against *S. aureus* with new modes of action. Antibacterial agents which primarily act as surfactants may have the potential of filling this need, since they may target the extracytoplasmic region and thus do not need to enter the cell, thereby avoiding most cellular pump-based resistance mechanisms. In our preliminary screening, dodecyl gallate was found to show antibacterial activity specifically against Gram-positive bacteria, while its parent compound, gallic acid did not show this activity, indicating that the alkyl group is related to the activity. This prompted us to test a series of alkyl gallates against methicillin resistant *S. aureus* (MRSA) strains in order to gain new insights into their antibacterial action on a molecular basis. The work has been communicated in part[6,7] and is described in full.

Results and Discussion

Antibacterial activity of propyl (C_3) (**1**), octyl (C_8) (**2**) and dodecyl (C_{12}) (**3**) gallates (see Fig. 1 for structures) against the six selected Gram-positive bacteria, *S. aureus*, *Bacillus subtilis*, *Brevibacterium ammoniagenes*, *Propionibacterium acnes*, *Streptococcus mutans*, and

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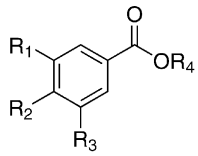
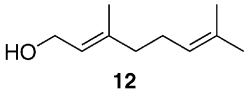
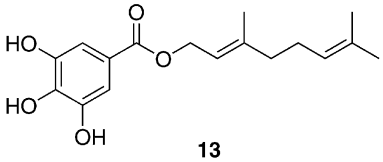


1: $R_1 = R_2 = R_3 = OH$, $R_4 = CH_2CH_2CH_3$
2: $R_1 = R_2 = R_3 = OH$, $R_4 = CH_2(CH_2)_6CH_3$
3: $R_1 = R_2 = R_3 = OH$, $R_4 = CH_2(CH_2)_{10}CH_3$
4: $R_1 = R_2 = R_3 = OH$, $R_4 = CH_2(CH_2)_7CH_3$
5: $R_1 = R_2 = R_3 = OH$, $R_4 = CH_2(CH_2)_8CH_3$
6: $R_1 = R_2 = R_3 = OH$, $R_4 = CH_2(CH_2)_9CH_3$
7: $R_1 = R_2 = R_3 = OH$, $R_4 = H$
8: $R_1 = R_2 = R_3 = OH$, $R_4 = CH_2(CH_2)_{12}CH_3$
9: $R_1 = R_2 = R_3 = H$, $R_4 = CH_2(CH_2)_9CH_3$
10: $R_1 = R_2 = OH$, $R_3 = H$, $R_4 = CH_2(CH_2)_7CH_3$
11: $R_1 = R_2 = OH$, $R_3 = H$, $R_4 = CH_2(CH_2)_8CH_3$



12



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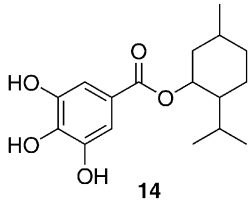

14

Figure 1. Chemical structures of alkyl gallates and related compounds.

Micrococcus luteus, is listed in Table 1. On the basis of this, a series of alkyl gallates (3,4,5-trihydroxybenzoates) was synthesized and tested for their antibacterial activity against the same Gram-positive bacteria (data not listed). All the bacteria tested are susceptible to alkyl gallates. Notably, the activity was not significantly increased as the carbon chain length increased. For example, octyl (C_8), nonyl (C_9) (**4**), decyl (C_{10}) (**5**), undecyl (C_{11}) (**6**) and dodecyl (C_{12}) gallates were all found to show the same MBC of 25 μg/mL against *M. luteus*. This inhibition pattern is similar in general against the other Gram-positive bacteria tested. The length of the alkyl chain does not appear to be a major contributor to the potency of their bactericidal activity, suggesting that the mode of antibacterial action of alkyl gallates may differ from those found for alkanols.[3]

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

Bacteria tested	MIC and *MBC* (μg/mL)		
	C_3	C_8	C_{12}
Bacillus subtilis	800 (*1600*)	12.5 (*25*)	12.5 (*25*)
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*)

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

Since discovery of effective antibacterial agents against *S. aureus* are urgently needed, further discussion is based on the data mainly against the methicillin resistant *S. aureus* (MRSA) ATCC 33591 strain as an example, unless otherwise specified. The antibacterial activity of the alkyl gallates against this MRSA strain is listed in Table 2. Notably, decyl (C_{10}), undecyl (C_{11}) and dodecyl (C_{12}) gallates were found to be the most effective with MBCs of 25 μg/mL. In contrast to their antifungal activity,[1] the length of the alkyl chain is not significantly related to the antibacterial activity. However, it must play a role in eliciting the activity since gallic acid (**7**) did not exhibit any bactericidal activity up to 3200 μg/mL. The MBC values of alkyl (C_8–C_{14}) gallates were in the range between 25 and 50 μg/mL. Differences in the minimum inhibitory concentrations (MIC) and MBC values against *S. aureus* were not more than 2-fold, indicating that their activity is bactericidal. In the case against *S. cerevisiae*, the antifungal activity of alkyl gallates was distinctly increased with each additional CH_2 group,[1] and the activity disappeared after the chain length reached the maximum activity. This is the so-called 'cutoff' which is a well known phenomenon. However, the clear cutoff was not observed with the same alkyl gallates against the bacteria. In the case against MRSA, tetradecyl (C_{14}) (**8**) gallate still showed the activity with a MBC of 50 μg/mL. Synthesis was achieved up to eicosanyl (C_{20}) gallate but the assay data were obtained unequivocally only up to tetradecyl (C_{14}) gallate because of solubility problems in the water based test medium.

The bactericidal effect of dodecyl gallate against *S. aureus* ATCC 33591 (MRSA) was confirmed by the time kill curve experiment as shown in Figure 2. Cultures of this MRSA strain, with a cell density of 1.0×10^5 colony forming units (CFU)/mL, were exposed to three different concentrations of dodecyl gallate. The number of

Table 2. Antibacterial activity of gallic acid and its esters against MRSA ATCC 33591

Compounds tested	μg/mL MIC (*MBC*)
Gallic acid	3200 (*>3200*)
C_3	1600 (*3200*)
C_6	50 (*200*)
C_7	25 (*100*)
C_8	25 (*50*)
C_9	25 (*50*)
C_{10}	12.5 (*25*)
C_{11}	12.5 (*25*)
C_{12}	12.5 (*25*)
C_{13}	50 (*50*)
C_{14}	50 (*50*)
Geranyl gallate	25 (*50*)
Menthyl gallate	25 (*50*)
Methicillin	800 (*>800*)

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

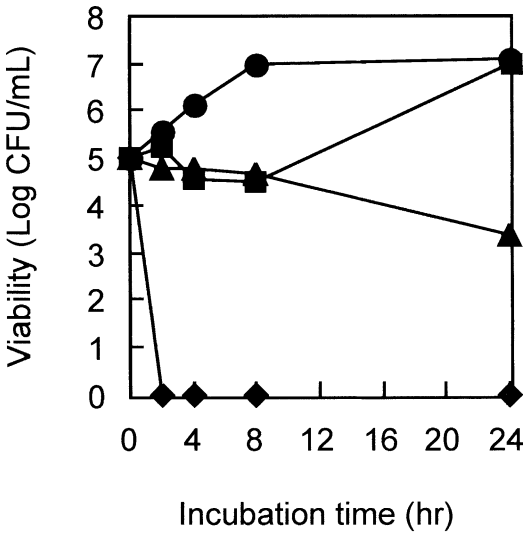

Figure 2. Bactericidal effects of dodecyl gallate against *S. aureus* ATCC 33591. Exponentially growing cells of *S. aureus* were inoculated into NYG broth and then cultured at 37 °C without shaking. Dodecyl gallate; 0 (●), 12.5 (■), 25 (▲), 50 (◆) μg/mL.

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 unlikely disrupts specific target proteins such as cell-surface receptors or signal transduction proteins.

On the basis of the results obtained, it appears that octyl and dodecyl gallates were bactericidal against MRSA ATCC 33591 strain at any growth stage. This activity was observed even in the chloramphenicol-treated cells, but the rate of decrease of cell number was slower than that in the exponentially growing cells.[6,7] The bactericidal activity of medium-chain alkyl gallates was noted in combination with their ability to disrupt the native membrane-associated function nonspecifically as surface-active agents (surfactants) and/or to inhibit the respiratory electron transport. The length of the alkyl chain is not a major contributor but plays an important role in eliciting the activity. For example, the amount of alkyl gallates entering into the cytosol or membrane lipid bilayer obviously regulates the activity.

The antibacterial activity of octyl and dodecyl gallates as well as gallic acid, penicillin G and methicillin were tested against the six selected *S. aureus* strains for comparison. The results are listed in Table 3. Both octyl and dodecyl gallates were effective against all the strains of *S. aureus* tested, with the MICs ranging from 12.5 to 50 μg/mL. The activity of dodecyl gallate is slightly more potent than that of octyl gallate against all the strains tested. Two strains of methicillin-resistant *S. aureus* were also resistant to penicillin G. Overall, the MICs of octyl and dodecyl gallates did not considerably differ among the strains tested.

The alkyl gallates can be considered as a head and tail structure, and hence its mode of antibacterial action was expected to act as surfactants. However, additional functions may need to be considered for the newly synthesized phenolic esters. For example, the ester group did not exist in the original alkanol structure and it may be related in eliciting the activity. Since alkanols themselves exhibit the antibacterial activity, the possibility of *S. aureus* exuding an esterase that hydrolyzes gallates to the original gallic acid and the corresponding alkanols, was first taken into account. This possibility can be readily ruled out since the MBC of undecyl gallate showed 25 μg/mL. More specifically, if undecyl gallate was hydrolyzed by the esterase exuded by *S. aureus*, the freed undecanol should show the activity with a MBC of 25 μg/mL,[3] but the freed gallic acid was found to possess no activity against *S. aureus* up to 3200 μg/mL. In addition, undecyl benzoate (**9**) did not exhibit any antibacterial activity against this bacterium up to 400 μg/mL.

The difference between antifungal and antibacterial actions of alkyl gallates needs to be considered. The head and tail structure of alkyl gallates is similar to alkanols of which primary antifungal action comes from their ability to disrupt the native membrane-associated function of the integral proteins as nonionic surfactants. In fact, amphipathic alkyl gallates were found to act primarily as surfactants against *S. cerevisiae*. However, the data obtained indicate that their antibacterial activity against *S. aureus* cannot explain that membrane damage alone is the cause of the lethal effect. The mode of the antibacterial action of alkyl gallates seems to differ from that of their antifungal action to some extent.

Notably, alkyl gallates were found to inhibit bacterial respiratory system. For example, dodecyl gallate inhibited the oxygen consumption of *Pseudomonas aeruginosa* IFO 3080 cells when the suspensions prepared from these bacterial cells were incubated with octyl or dodecyl gallates. In addition, octyl and dodecyl gallates inhibited *P. aeruginosa* NADH oxidase by a membrane fraction prepared from the same bacterial cells. The assay was carried out as previously described,[8–10] and the results observed indicate that alkyl gallates inhibits the bacterial membrane respiratory chain.[11] It should be noted that *P. aeruginosa* IFO 3080 strain used for the experiment is a strict aerobic bacterium. The respiratory inhibition causes the bacterial cell death for the lack of anaerobic fermentative ability. Apparently, the antibacterial activity of alkyl gallates comes, at least in part, from their ability to inhibit respiratory chain enzyme activity. Dodecyl gallates also inhibited the oxygen consumption of *M. luteus* ATCC 4698 cells when the suspensions prepared from the same bacterial cells were incubated with this gallate. The inhibition by dodecyl gallates showed dose–response and the concentration found to inhibit oxygen consumption was approximately comparable to that causing bactericidal activity against the Gram-positive bacteria tested, except *S. mutans*.

Table 3. Antibacterial activity of octyl and dodecyl gallates against the six selected strains of *Staphylococcus aureus*

Compounds tested	MIC and *MBC* (μg/mL)					
	ATCC 12598[a]	ATCC 25923[a]	ATCC 33591[b]	ATCC 33592[b]	ATCC 11632[c]	ATCC 29247[c]
Penicillin G	0.049 (—)	0.049 (—)	>800 (—)	>800 (—)	800 (—)	>800 (—)
Methicillin	1.56 (>*6.25*)	1.56 (—)	800 (>*800*)	800 (>*800*)	1.56 (—)	3.13 (*50*)
Octyl gallate	25 (*50*)	25 (—)	25 (*50*)	50 (*50*)	25 (—)	25 (—)
Dodecyl gallate	12.5 (*25*)	12.5 (*12.5*)	12.5 (*25*)	25 (*25*)	12.5 (*25*)	12.5 (*25*)
Gallic acid	>3200 (—)	>3200 (—)	3200 (>*3200*)—	—	—	

—, Not tested. Numbers in *Italic* type in parentheses are *MBC*.
[a]Methicillin-susceptible.
[b]Methicillin-resistant.
[c]Penicillin-resistant *S. aureus*.

It is obvious that the pyrogallol moiety plays an important role in eliciting activities. This hydrophilic group first binds with an intermolecular hydrogen bond like a 'hook' attaching itself to the hydrophilic portion of the membrane. The hydrophobic alkyl portion of the molecule is then able to enter into the membrane lipid bilayers where various enzymes, especially components of energy converting systems such as electron transport chains (ETCs) and ATPases are embedded. An ETC is a chain of specialized molecules (redox agents), which form a conducting path for electrons. It should be borne in mind however that the ETC involved in the respiratory chain occurs at the inner face of the cytoplasmic membrane. On the basis of the data obtained, it can be assumed that the amphipathic alkyl gallates enter into the lipid bilayers[12] and inhibit the ETC, perhaps by interfering with 'redox' reactions. This may reveal why alkyl gallates do not show a clear cutoff in their antibacterial activity. The pyrogallol moiety apparently plays a major role in this interference. The conclusion can be supported by the fact that alkyl gallates are known as antioxidants.[13,14] For example, the same alkyl gallates scavenge DPPH radical and superoxide anion generated by xanthine oxidase.[15] The possibility that alkyl gallates disrupt in part the membrane-associated functions of respiration related enzymes as nonionic surfactants cannot be ruled out. Taking altogether, dodecyl gallate first acts as a surfactant and subsequently inhibits the ETC. Dodecyl gallate unlikely disrupts specific target proteins such as cell-surface receptors or signal transduction proteins. In brief, alkyl gallates do not act by a single defined process but has multiple functions. The amount of alkyl gallates entering into the cytosol or lipid bilayer is dependent on the alkyl chain length. The rationale for this still remains largely unclear.

Alkyl gallates were described to induce apoptosis in human leukemia HL60 RG and to show cytotoxic effects on other cell lines.[16–19] In these apoptotic processes, the generation of ROS (reactive oxygen species) is thought to contribute in the initiation of apoptosis.[17,20,21] Membrane lipids are abundant in unsaturated fatty acids, and 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. Alkyl gallates were found to induce cellular ROS generation. However, ROS generation in *S. aureus* cells caused by octyl and dodecyl gallates is not directly associated with their bactericidal action since the antioxidants such as α-tocopherol, L-ascorbate, and *N*-acetylcysteine did not exhibit any protective effect. The antibacterial activity of these gallates is not due to the pro-oxidant action. Rather, octyl and dodecyl gallates act as antioxidants and protect from oxidative damage, similar to those described.[22] It should be noted that oxidative stress is derived from ROS produced via oxidative phosphorylation in ETC and hence, octyl and dodecyl gallates can be considered to reach the inner face of cytoplasmic membrane and influence the functions relating ROS generation.[6,7]

It is apparent that alkyl gallates do not act by a single defined process but rather has multiple functions such as nonionic surfactants and respiratory inhibitors by which they exert their bactericidal and fungicidal action. On the basis of the data obtained so far, it may be logical to conclude that respiratory inhibition plays a more essential role in antibacterial activity of alkyl gallates against Gram-positive bacteria. In contrast, surfactant process may be a major contributor to their antifungal activity at least against *S. cerevisiae*. In either case, medium chain alkyl gallates first act as surfactants and are then involved in biochemical processes more or less. The antibacterial mechanism of alkyl gallates is largely associated with their specific pyrogallol structure, and the length of the alkyl chain also plays a role in eliciting the activity to some extent. For example, octyl gallate exhibited a broad antimicrobial spectrum including antifungal and antibacterial activity[1] whereas dodecyl gallate showed only antibacterial activity specifically against Gram-positive bacteria.

The data obtained were compared with those of alkanols.[3] The potency of the antibacterial activity of alkanols against *S. aureus* was distinctly increased with each additional CH_2 group up to undecanol. The increased lipophilicity of alkanols should affect their movement across the membrane or into the lipid bilayer, depending on the alkyl chain length. In any case, alkanols are chemically stable compounds and may not react with any biologically important substances in the cytosol or lipid bilayer. The primary antibacterial action of alkanols should come from their ability to function as nonionic surfactants.[23] It is obvious that the bactericidal

action of alkyl gallates is similar to those of alkanols in many aspects but differs to some extent. The data obtained so far indicates that the hydroxyl group of alkanols can be replaced by any hydrophilic groups as long as the 'head and tail' structure is balanced. For example, we became aware that nonyl protocatechuate (3,4-dihydroxybenzoate) was effective against *S. aureus* ATCC 33591 during our previous study.[1] Hence, the same series of alkyl protocatechuates was synthesized in the same manner and assayed against the same MRSA strain for comparison. The results are listed in Table 4. The data obtained for alkyl protocatechuates are similar to those found for alkyl gallates. The activity was not significantly increased with each additional CH_2 group. Among the alkyl protocatechuates tested, both nonyl (**10**) and decyl (**11**) protocatechuates were found to be the most potent each with a MBC of 50 μg/mL. The bactericidal effect of nonyl protocatechuate against *S. aureus* ATCC 33591 was confirmed by the time-kill curve experiment, similar to those found for dodecyl gallate.[1] The same alkyl protocatechuates were also effective against methicillin susceptible *S. aureus* ATCC 6538, and the results obtained (data not listed) are similar to those found against the MRSA strain. Nonyl protocatechuate also inhibited the oxygen consumption of *P. aeruginosa* IFO 3080 cells. In addition, nonyl protocatechuate inhibited *P. aeruginosa* NADH oxidase by a membrane fraction prepared from the same bacterial cells.

The introduction of branching or unsaturation into the hydrophobic group is known to increase the solubility of the surfactant in water.[24] If 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. In addition, alkyl gallates act as multifunctional agents—at least as antimicrobial and antioxidant agents.[22] The multifunction concept has been further extended to geranyl gallate since geraniol (**12**) has previously been reported to increase glutathione *S*-transferase activity, which is believed to be a major mechanism for chemical carcinogen detoxification.[25] Based on the above concept, both geranyl gallate (**13**) and menthyl gallate (**14**) were synthesized and tested for their antibacterial activity against *S. aureus* ATCC 33591. As a result, these gallates exhibited the activity but did not increase it as much as expected (Table 2). After geranyl gallate is consumed together with the food, this ester is hydrolyzed to the gallic acid and geraniol, which are common plant components. The freed gallic acid acts as an antioxidant and geraniol induces glutathione *S*-transferase activity. Similarly, various bulky alkyl protocatechuates, *trans*-2-nonene-1-yl (**15**), *cis*-2-nonene-1-yl (**16**), 2-nonanyl (**17**), 3-nonanyl (**18**), cyclohexylmethyl (**19**), geranyl (**20**), neryl (**21**), decahydro-2-naphtyl (**22**), menthyl (**23**), and bornyl (**24**) derivatives were synthesized in the same manner and assayed against the same MRSA strain for comparison (Fig. 3). The results are listed in Table 5. All the protocatechuates tested, regardless of their alkyl shape, exhibited the activity but not as much as expected. The MBC to MIC ratio of cyclohexylmethyl protocatechuate is 8-fold. As far as alkyl protocatechuates are compared, the compounds possessing the similar log *P* values exhibit the similar MBC values, and 25 μg/mL seems to be the maximum activity through synthetic optimization, as far as alcohols are concerned as a hydrophobic portion. Similar to geranyl gallate, geranyl protocatechuate is hydrolysable to protocatechuic acid and geraniol, which are common plant components. The freed protocatechuic acid acts as an antioxidant[26] and geraniol induces glutathione

Table 4. Antibacterial activity of protocatechuic acid and its esters against MRSA ATCC 33591

Compounds tested	μg/mL MIC (*MBC*)	log *P*
Protocatechuic acid	3200 (*>3200*)	
C_3	800 (*1600*)	1.90
C_4	400 (*800*)	2.32
C_5	200 (*200*)	2.73
C_6	50 (*100*)	3.15
C_7	50 (*100*)	3.57
C_8	50 (*100*)	3.99
C_9	50 (*50*)	4.40
C_{10}	25 (*50*)	4.82
C_{11}	25 (*100*)	5.24
C_{12}	25 (*>800*)	5.65
C_{13}	50 (*>800*)	6.07
C_{14}	>800 (*>800*)	6.49

Numbers in *Italic* type in parentheses are *MBC*. Log *P* values were calculated by the method described.

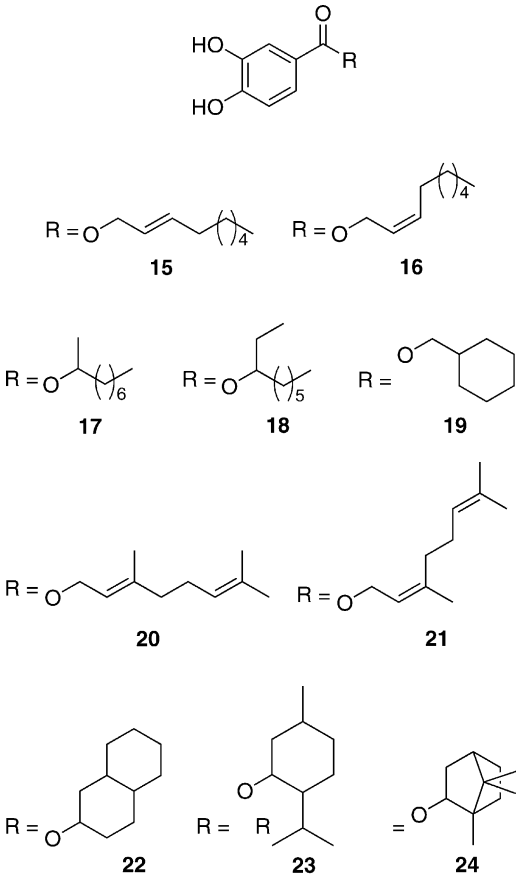

Figure 3. Chemical structures of alkyl protocatechuates and related compounds.

Table 5. Antibacterial activity of bulky alkyl protocatechuates against MRSA ATCC 33591

Compounds tested	μg/mL MIC (*MBC*)	log *P*
trans-2-Nonene-1-yl (**15**)	25 (*50*)	4.22
cis-2-Nonene-1-yl (**16**)	25 (*50*)	4.22
2-Nonanyl (**17**)	25 (*50*)	4.30
3-Nonanyl (**18**)	25 (*50*)	4.37
Cyclohexylmethyl (**19**)	25 (*200*)	3.05
Geranyl (**20**)	25 (*25*)	3.84
Neryl (**21**)	25 (*100*)	3.84
Decahydro-2-naphtyl (**22**)	25 (*100*)	3.62
Menthyl (**23**)	12.5 (*50*)	4.10
Bornyl (**24**)	12.5 (*25*)	3.78

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

S-transferase activity. It seems that various biological activities needed for food protection are combined by selecting appropriate head and tail portions.

The 'hydrolysable' ester group was purposely selected in order to prevent undesired side effects; particularly the endocrine-disrupting activity of environmentally persistent estrogen mimics[27] such as alkylphenolic compounds.[28] Propyl, octyl and dodecyl gallates are currently permitted for use as antioxidant additives in food.[13] In connection with food, 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*. In addition to their potent antioxidant activity,[15] the bactericidal activity of octyl and dodecyl gallates reported here, especially anti-MRSA activity should be their additional benefit. Interestingly, gallic acid was previously reported to inhibit the growth of harmful intestinal bacteria such as *Clostridium perfringens*, *C. paraputrificum*, *S. aureus* and *Escherichia coli*, but not beneficial intestinal bacteria such as *Bifidobacterium adolescentis* and *Lactobacillus acidophilus*.[29] The results obtained seem to provide a more rational and scientific approach to design selective and effective antibacterial agents.

Experimental

Chemicals

A series of each alkyl gallates and protocatechuates used for the assay were available from our previous work.[2] Propyl, octyl and dodecyl gallates were purchased from Aldrich Chemical Co. (Milwaukee, WI). Methicillin and chloramphenicol were obtained from Sigma Chemical Co. (St. Louis, MO). Geraniol, *trans* 2-nonene-1-ol, and *cis* 2-nonene-1-ol were obtained from Alfa Aesar (Ward Hill, MA). 3-Nonanol was purchased from TCI America (Portland, OR). Other primary and secondary alcohols, protocatechuic acid, gallic acid, and *N,N′*-dicyclohexylcarbodiimide (DCC) were obtained from Aldrich. For the experiment, all compounds were first dissolved in *N,N*-dimethylformamide (DMF) that was purchased from EM Science (Gibbstown, NJ). The concentration of DMF in each medium was always 1%.

Synthesis

To a solution of protocatechuic acid (1.3 mmol) and alcohol (1.3 mmol) in THF (10 mL) cooled at 0 °C was added 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 $MgSO_4$ and evaporated. The crude products were purified by chromatography (SiO_2; elution with 40% AcOEt-*n*-hexane). Their various analogues (**14**–**24**) were also synthesized in the same manner. Structures of the synthesized esters were established by spectroscopic methods (IR, MS, and NMR).[2] Log *P* values were achieved by ChemDraw Pro version 4.5 (CambridgeSoft CO. Cambridge, MA) using Crippen's fragmentation.[30]

Menthyl gallate (14). This was obtained in 46% yield as a colorless solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.76 (d, J = 7.0 Hz, 3H), 0.90 (d, J = 7.0 Hz, 3H), 0.92 (d, J = 7.0 Hz, 3H), 1.10 (m, 2H), 1.52 (m, 2H), 1.72 (m, 2H), 1.93 (m, 1H), 2.07 (m, 1H), 4.86 (dt, J = 3.6, 8.8 Hz), 6.82 (bs, 3H), 7.32 (s, 2H). IR (Nujol) 3345, 2930, 1670, 1545, 1450, 1325, 1030 cm^{-1}. FAB-MS (*m*/*z*) 309 ($M + H^+$).

***trans*-2-Nonene-1-yl 3,4-dihydroxybenzoate (15).** This was obtained in 52% yield as a colorless powder. ^{1}H NMR (400 MHz, CD_3COCD_3) δ 0.86 (t, J = 7.2 Hz, 3H), 1.31 (m, 8H), 2.07 (quar, J = 6.8 Hz, 2H), 4.67 (ddd, J = 0.8, 1.2, 6.8 Hz, 2H), 5.68 (ttd, J = 1.2, 6.8, 12.8 Hz, 1H), 5.86 (ttd, J = 0.8, 6.8, 12.8 Hz, 1H), 6.89 (d, J = 8.4, 1H), 7.44 (dd, J = 1.6, 8.4, 1H), 7.50 (d, J = 1.6, 1H), 8.47 (bs, 2H); ^{13}C NMR (100 MHz, CD_3COCD_3) δ 14.3, 23.2, 29.5, 29.7, 32.4, 32.9, 65.5, 115.6, 117.0, 122.8, 123.1, 125.2, 136.2, 145.3, 150.5, 166.0. IR (Nujol) 3490, 3330, 1680, 1610, 1295, 1230, 1100, 960 cm^{-1}. EI–MS, *m*/*z* 278 (M^+).

***cis*-2-Nonene-1-yl 3,4-dihydroxybenzoate (16).** This was obtained in 55% yield as a colorless powder. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.87 (t, J = 6.8 Hz, 3H), 1.30 (m, 8H), 2.15 (quar, J = 6.8 Hz, 2H), 4.85 (d, J = 6.4 Hz, 2H), 5.67 (m, 2H), 6.38 (bs, 2H). 6.91 (d, J = 8.0, 1H), 7.57 (dd, J = 2.0, 8.0, 1H), 7.70 (d, J = 2.0, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.2, 22.7, 27.7, 29.0, 29.5, 31.8, 61.1, 114.7, 116.6, 122.3, 122.9, 123.8, 135.8, 143.0, 148.8, 166.9. IR (Nujol) 3490, 3340, 1680, 1610, 1295, 1225, 1100 cm^{-1}. EI–MS, *m*/*z* 278 (M^+).

2-Nonanyl 3,4-dihydroxybenzoate (17). This was obtained in 25% yield as a colorless powder. ^{1}H NMR (400 MHz, CD_3COCD_3) δ 0.85 (t, J = 6.4 Hz, 3H), 1.27 (d, J = 6.4 Hz, 3H), 1.29 (m, 10H), 1.64 (m, 2H), 5.04 (sex, J = 6.4 Hz, 1H), 6.88 (d, J = 8.4, 1H), 7.43 (dd, J = 2.0, 8.4, 1H), 7.50 (d, J = 2.0, 1H), 8.43 (bs, 2H); ^{13}C NMR (100 MHz, CD_3COCD_3) δ 14.3, 20.4, 23.3, 26.2, 29.9, 30.1, 32.5, 36.7, 71.3, 115.5, 116.9, 123.0, 123.4, 145.3, 150.3, 165.9. IR (Nujol) 3490, 3350, 1690, 1610, 1295, 1230, 1100 cm^{-1}. EI–MS, *m*/*z* 280 (M^+).

3-Nonanyl 3,4-dihydroxybenzoate (18). This was obtained in 25% yield as a colorless oil. ^{1}H NMR (400 MHz, CD_3COCD_3) δ 0.85 (t, J = 7.2 Hz, 3H), 0.91 (d, J = 7.6 Hz, 3H), 1.27 (m, 8H), 1.65 (m, 4H), 4.97 (quin, J = 6.8 Hz, 1H), 6.89 (d, J = 8.4, 1H), 7.45 (dd, J = 2.4, 8.4, 1H), 7.50 (d, J = 2.4, 1H), 8.48 (bs, 2H); ^{13}C NMR (100 MHz, CD_3COCD_3) δ 10.0, 14.3, 23.2, 26.1, 27.8, 29.9, 32.5, 34.4, 75.7, 115.5, 116.9, 123.0, 123.3, 145.3, 150.3, 166.1. IR (neat) 3340, 2940, 1675, 1600, 1445, 1295, 1235, 1110 cm^{-1}. EI–MS, m/z 280 (M^+).

Cyclohexylmethyl 3,4-dihydroxybenzoate (19). This was obtained in 65% yield as a colorless powder. ^{1}H NMR (400 MHz, CD_3COCD_3) δ 1.05–1.28 (m, 5H), 1.68 (m, 6H), 4.03 (d, J = 6.4 Hz, 2H), 6.89 (d, J = 8.4, 1H), 7.45 (dd, J = 2.0, 8.4, 1H), 7.50 (d, J = 2.0, 1H), 8.47 (bs, 2H); ^{13}C NMR (100 MHz, CD_3COCD_3) δ 26.3, 26.4, 27.0, 38.2, 69.8, 115.4, 116.8, 122.9, 123.0, 145.2, 155.5, 166.2. IR (Nujol) 3490, 3330, 1680, 1605, 1300, 1230, 1110 cm^{-1}. EI–MS, m/z 250 (M^+).

Geranyl 3,4-dihydroxybenzoate (20). This was obtained in 65% yield as a colorless powder. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.60 (s, 3H), 1.67 (d, J = 0.4 Hz, 3H), 1.75 (s, 3H), 2.07 (m, 4H), 4.81 (d, J = 6.8 Hz, 2H), 5.09 (m, 1H), 5.44 (dt, J = 1.2, 6.8 Hz, 1H), 6.53 (bs, 2H), 6.90 (d, J = 8.0, 1H), 7.57 (dd, J = 2.0, 8.0, 1H), 7.71 (d, J = 2.0, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 16.5, 17.7, 25.6, 26.2, 39.5, 62.0, 114.6, 116.5, 117.9, 122.3, 123.4, 123.5, 131.6, 142.3, 142.9, 148.6, 166.9. IR (Nujol) 3510, 3300, 1690, 1610, 1360, 1295, 1220, 1125, 980 cm^{-1}. EI–MS, m/z 288 (M^+-2H).

Neryl 3,4-dihydroxybenzoate (21). This was obtained in 57% yield as a colorless powder. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.60 (s, 3H), 1.67 (s, 3H), 1.75 (d, J = 0.8 Hz, 3H), 2.13 (m, 4H), 4.78 (d, J = 7.2 Hz, 2H), 5.11 (m, 1H), 5.46 (m,1H), 6.37 (bs, 1H), 6.70 (bs, 1H), 6.90 (d, J = 8.0, 1H), 7.56 (dd, J = 2.0, 8.0, 1H), 7.70 (d, J = 2.0, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 17.6, 23.5, 25.6, 26.6, 32.2, 61.7, 114.5, 116.5, 118.8, 122.3, 123.3, 123.5, 132.0, 142.6, 142.9, 148.6, 166.9. IR (Nujol) 3500, 3300, 1680, 1610, 1295, 1190, 950, 775 cm^{-1}. EI–MS, m/z 288 (M^+-2H).

Decahydro-2-naphthyl 3,4-dihydroxybenzoate (22). This was obtained in 42% yield as a colorless powder. ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.80–2.10 (m, 16H), 4.90–5.20 (m, 1H), 6.18 (bs, 2H), 6.95 (m, 1H), 7.56 (m, 1H), 7.70 (m, 1H). IR (Nujol) 3460, 3280, 1680, 1605, 1290, 1240, 1120 cm^{-1}. FABMS, m/z 291 ($M+H^+$).

Menthyl 3,4-dihydroxybenzoate (23). This was obtained in 27% yield as a colorless powder. ^{1}H NMR (400 MHz, CD_3COCD_3) δ 0.78 (d, J = 6.8 Hz, 3H), 0.91 (d, J = 6.8 Hz, 3H), 0.92 (d, J = 6.8 Hz, 3H), 0.94 (m, 1H), 1.07 (m, 1H), 1.15 (m, 1H), 1.55 (m, 2H), 1.72 (m, 2H), 1.95 (dsep, J = 3.2, 7.2, 1H), 2.04 (m, 1H), 4.84 (dt, J = 4.4, 10.8, 1H), 6.89 (d, J = 8.4, 1H), 7.45 (dd, J = 2.0, 8.4, 1H), 7.51 (d, J = 2.0, 1H), 8.57 (bs, 2H); ^{13}C NMR (100 MHz, CD_3COCD_3) δ 16.8, 21.0, 22.3, 24.3, 27.2, 32.1, 35.0, 41.8, 48.1, 74.3, 115.5, 116.9, 123.0, 123.2, 145.3, 150.4, 165.7. IR (Nujol) 3440, 3360, 1700, 1610, 1300, 1190, 1115 cm^{-1}. EI–MS, m/z 292 (M^+).

Bornyl 3,4-dihydroxybenzoate (24). This was obtained in 32% yield as a colorless solid. ^{1}H NMR (400 MHz, CD_3COCD_3) δ 0.90 (s, 3H), 0.93 (s, 3H), 0.97 (s, 3H), 1.07 (dd, J = 3.2, 13.2 Hz, 1H), 1.32 (ddd, J = 4.4, 9.6, 12.0 Hz, 1H), 1.42 (ddt, J = 2.4, 4.4, 12.0 Hz, 1H), 1.72 (t, J = 4.4, Hz, 1H), 1.82 (dtt, J = 3.2, 4.4, 12.0 Hz, 1H), 2.15 (ddd, J = 4.4, 9.6, 12.0 Hz, 1H), 2.42 (dddd, J = 3.2, 4.4, 9.6, 13.2 Hz, 1H), 5.04 (ddd, J = 2.4, 3.2, 9.6 Hz, 1H), 6.91 (d, J = 8.4, 1H), 7.48 (dd, J = 2.0, 8.4, 1H), 7.90 (d, J = 2.0, 1H), 8.52 (bs, 2H); ^{13}C NMR (100 MHz, CD_3COCD_3) δ 13.9, 19.1, 20.0, 28.0, 28.6, 37.6, 45.7, 48.4, 49.6, 80.0, 115.6, 116.8, 123.0, 123.2, 145.3, 150.4, 166.4. IR (Nujol) 3380, 1670, 1610, 1300, 1230, 1100 cm^{-1}. EI–MS, m/z 290 (M^+).

Test strains. The microorganisms, *Bacillus subtilis* ATCC 9372, *Brevibacterium ammoniagenes* ATCC 6872, *Micrococcus luteus* ATCC 4698, *Streptococcus mutans* ATCC 25175, *Propionibacterium acnes* ATCC 11827, *Staphylococcus aureus* ATCC 12598, *S. aureus* ATCC 25923, *S. aureus* ATCC 33591 (methicillin resistant), *S. aureus* ATCC 33592 (gentamicin and methicillin resistant), *S. aureus* ATCC 11632 (penicillin resistant), *S. aureus* ATCC 29247, and *S. aureus* ATCC 6538, were purchased from American Type Culture Collection (Manassas, VA). *Pseudomonas aeruginosa* IFO 3080 was available from our previous works.[8–10]

Medium. The NYG culture medium for the bacteria consisted of 0.8% nutrient broth (BBL, BD, Franklin Lakes, NJ), 0.5% yeast extract (DIFCO, BD, Franklin Lakes, NJ), and 0.1% glucose.

Antibacterial assay. Broth macrodilution methods were used as previously described[2,3] 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 a 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 showed no visible growth in the drug-free cultivation. The assays were performed at least in triplicate on separate occasions.

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.

Measurement of reactive oxygen species (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.[31] 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 Cytofluor 2300 fluorescence spectrophotometer (Millipore Co.) with excitation at 480 nm and emission at 530 nm. The arbitrary units were based directly on fluorescence intensity.

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