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Anti-MRSA Activity of Alkyl Gallates

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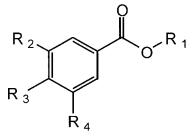
Abstract—A series of alkyl gallates (3,4,5-trihydroxybenzoates) was found to show antibacterial activity against Gram-positive bacteria including methicillin resistant *Staphylococcus aureus* (MRSA) strains. For example, dodecyl (C_{12}) gallate (**1**) exhibited bactericidal activity against MRSA ATCC 33591 strain with the minimum bactericidal concentration (MBC) of 25 μg/mL (74 μM). The time–kill curve study showed that dodecyl gallate is bactericidal against this MRSA strain. This bactericidal activity comes in part from its ability to inhibit respiratory electron transport systems. The length of the alkyl chain is not a major contributor but plays an important role in eliciting the activity.

In our previous paper, we reported antifungal activity of octyl (C_8) gallate (**2**) (see Fig. 1 for structures) against *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, *Candida albicans* and *Aspergillus niger*.[1] Among propyl (C_3) (**3**), octyl and dodecyl gallates that are currently permitted for use as antioxidant additives in food,[2] octyl gallate was noted to be the only active compound against fungi. The primary antifungal activity of octyl gallate against these fungi comes from its ability to act as a nonionic surface-active agent (surfactant), similar to alkanols.[3] During this study we became aware that dodecyl gallate still exhibits antibacterial activity specifically against Gram-positive bacteria. As far as antibacterial activity is compared, dodecyl gallate is even slightly more potent than that of octyl gallate. The result indicates that the length of the alkyl group is obviously related to the activity since these gallates possess the same hydrophilic head portion.

Due to the adaptability, *Staphylococcus aureus* can easily develop resistance to commonly used antibiotics. This resistance involves the enzymic inactivation in resistant bacteria. Such resistance genes are often transferred to other bacteria by a variety of gene transfer mechanisms.[4] 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 target the extracytoplasmic region and thus do not need to enter the cell, thereby avoiding most cellular pump-based resistance mechanisms. The ideal antimicrobial agent was recently suggested to produce by rational design.[5] This prompted us to investigate dodecyl gallate to gain new insights into its selective antibacterial action on a molecular basis.

Dodecyl (lauryl) gallate was first tested for its antibacterial activity against the seven selected Gram-positive bacteria. The results are listed in Table 1. All of the bacteria tested were found to be susceptible to dodecyl gallate.[6] Among them, *Propionibacterium acnes* is the most susceptible to this gallate with an MBC of 6.25 μg/mL (18 μM). Subsequently, MBCs against *S. aureus*, *Bacillus subtilis*, *Micrococcus luteus*, and *Brevibacterium ammoniagenes* were 25 μg/mL (74 μM), and against *Streptococcus mutans* was 100 μg/mL (296 μM), respectively. It should be noted that dodecyl gallate was equally effective against all the *S. aureus* tested, including two MRSA strains.



1. $R_1 = (CH_2)_{11}CH_3$, $R_2 = R_3 = R_4 = OH$
2. $R_1 = (CH_2)_7CH_3$, $R_2 = R_3 = R_4 = OH$
3. $R_1 = (CH_2)_2CH_3$, $R_2 = R_3 = R_4 = OH$
4. $R_1 = (CH_2)_9CH_3$, $R_2 = R_3 = R_4 = OH$
5. $R_1 = (CH_2)_{10}CH_3$, $R_2 = R_3 = R_4 = OH$
6. $R_1 = (CH_2)_{10}CH_3$, $R_2 = OH$, $R_3 = R_4 = H$
7. $R_1 = (CH_2)_{10}CH_3$, $R_2 = R_4 = OH$, $R_3 = H$

Figure 1. Chemical structures of gallates and related compounds.

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Table 1. Antibacterial activity (μg/mL) of dodecyl gallate

Bacteria tested	MIC	MBC
Bacillus subtilis	25	25
Bacillus thuringiensis	50	—[a]
Brevibacterium ammoniagenes	25	25
Micrococcus luteus	12.5	25
Streptococcus mutans	100	100
Propionibacterium acnes	6.25	6.25
Staphylococcus aureus	12.5	25
Staphylococcus aureus (MRSA)	12.5	25

[a]Not tested.

Since discovery of effective antibacterial agents against *S. aureus* are urgently needed, the current study was targeted mainly against MRSA ATCC 33591 strain as an example unless otherwise specified. A series of alkyl gallates was synthesized and tested for their antibacterial activity against this MRSA strain for comparison. The results are listed in Table 2. Notably, the activity was not significantly increased for every additional CH_2 group. For example, decyl (C_{10}) (**4**), undecyl (C_{11}) (**5**), and dodecyl (C_{12}) gallates were found to be the most effective with MBCs of 25 μg/mL. The length of the alkyl chain did not appear to be a major contributor to the potency of their bactericidal activity. Nonetheless, the alkyl group must play a role in eliciting the activity since gallic acid itself did not exhibit any bactericidal activity up to 3200 μg/mL. This inhibition pattern is similar in general against the other Gram-positive bacteria tested. The MBC values of gallates (C_8–C_{14}) were in the range between 25 and 50 μg/mL.[7,8] The differences in the minimum inhibitory concentrations (MICs) and MBCs against *S. aureus* were generally not more than 2-fold, indicating that no residual bacteriostatic activity is involved.

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 6.5×10^6 colony forming units (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. It shows that dodecyl gallate at MBC rapidly reduced the number of viable cells within the first hour and then slowed thereafter. The final cell count at MBC of dodecyl gallate was $1/10^4$ of the control, indicating that dodecyl gallate at MBC was bactericidal. Complete lethality occurred at 2×MBC. No viable cells were detected after being exposed to 50 μg/mL (2×MBC) of dodecyl gallate for 8 h. The result indicates that the amount of the drug's molecules needed to be increased with increasing number of viable cells. It seems that dodecyl gallate unlikely disrupts specific target proteins such as cell-surface receptors or signal transduction proteins.

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
Methicillin	800	>800

In the current study, the 'hydrolyzable' ester group was selected in order to avoid undesired side effects, particularly endocrine disrupting activity of environmentally persistent estrogen mimics[9] such as alkylphenolic compounds.[10] This newly introduced ester group in alkyl gallates needs to be checked if this group is related to the activity since alkanols themselves exhibit the antibacterial activity. For example, undecanol was described to be bactericidal against *S. aureus* with an MBC of 25 μg/mL (145 μM).[3] The possibility of *S. aureus* exuding an esterase that hydrolyzes alkyl gallates to gallic acid and the corresponding alcohols, may need to be taken into account. This possibility can be readily ruled out since none of undecyl benzoate, undecyl 3-hydroxybenzoate (**6**) or undecyl 3,5-dihydroxybenzoate (**7**), exhibited any antibacterial activity against *S. aureus* up to 400 μg/mL.

During the study to clarify modes of the antibacterial action, alkyl gallates were noted to inhibit bacterial respiratory systems. For example, dodecyl gallate inhibited the oxygen consumption of *Pseudomonas aeruginosa* cells when the suspensions prepared from the same bacterial cells were incubated with dodecyl gallate. It showed dose–response for this respiratory inhibition. Dodecyl gallate also inhibited *P. aeruginosa* NADH oxidase by a membrane fraction prepared from the same bacterial cells. This action does not affect directly on ATP synthetase but earlier in the electron transport

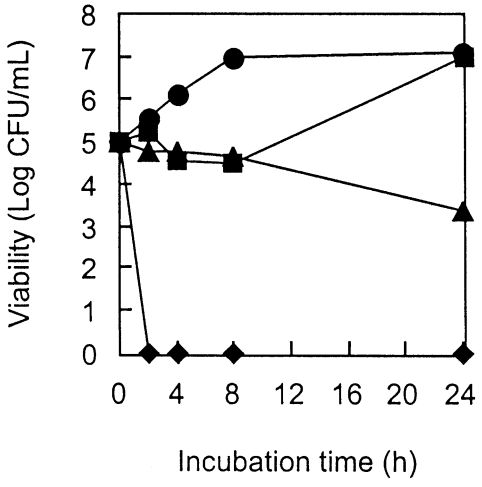

Figure 2. Effects of dodecyl gallate on the viability of *S. aureus* ATCC 33591. The cells of *S. aureus* were inoculated in NYG medium with 0 (●), 12.5 (■), 25 (▲), 50 (◆) μg/mL of dodecyl gallate at 37 °C.

chain (ETC), similar to alkanols.[11] It seems that the antibacterial activity of alkyl gallates comes at least in part from their ability to inhibit respiratory systems. The strain of *P. aeruginosa* IFO 3080 was used in the current study as previously described.[12–15] In connection with this, 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.[16] It should be noted that this IFO 3080 strain is not susceptible to most antibiotics from microbial origin but is sensitive to some antibacterial phytochemicals.[13,14] The concentrations of dodecyl gallate found to inhibit respiration are approximately comparable to those causing bactericidal activity against the bacteria tested, except *S. mutans*.

The difference between antibacterial and antifungal action of alkyl gallates needs to be taken into account. Their head and tail structure is similar to that of alkanols of which primary antifungal action comes from their ability to disrupt the native membrane-associated function of the integral proteins as nonionic surfactants.[3] In fact, amphiphilic gallates such as octyl and nonyl gallates were found to act primarily as surfactants against *S. cerevisiae*.[1] However, the data obtained so far indicates that their antibacterial activity is unlikely due to their surfactant property, although this concept cannot be entirely ruled out. Prokaryotic and eukaryotic microorganisms are known to differ in many ways. For example, the ETC involved in the respiratory systems 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 considered because the process by which gallates reach the action sites in 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, so the increased lipophilicity of the gallates should affect their movement more into the membrane lipid bilayer portions. The most molecules of the highly lipophilic dodecyl gallate being dissolved in the medium are incorporated into the lipid bilayers.[17] Once inside the lipid bilayer portions, gallates may inhibit the ETC, perhaps by interfering with the redox reactions. The pyrogallol moiety apparently plays a major role for the interference. This postulate can be supported by the fact that the gallates are known as antioxidants.[2,18] For example, the same gallates scavenge DPPH radical and superoxide anion generated by xanthine oxidase.[19] In addition, antimicrobial activity of phenolic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) is known.[20] In the case against fungi, dodecyl gallate can rarely enter into the cytosol and hence, cannot reach the mitochondria. This may explain why the gallates of which alkyl group is longer than decyl (C_{10}) did not show any effects on eukaryotic microorganisms such as *S. cerevisiae*. It is obvious that microorganisms having different membrane structure show different susceptibilities to gallates having different alkyl chain lengths. The hydrophobicity of molecules is well known to be associated with biological activity.[21] However, the rationale for this, especially the role of the hydrophobic portion, is still poorly understood because this portion has been given little attention.

It appears that alkyl gallates do not act by a single defined process but rather have multifunction such as nonionic surfactants and respiratory inhibitors by which they exert their fungicidal and/or bactericidal action. On the basis of the data obtained so far, it may be logical to conclude that biochemical mechanisms (respiratory inhibition) may play a more essential role in antibacterial activity of alkyl gallates against Gram-positive bacteria. In contrast, biophysical processes (surfactant action) are a major contributor to their antifungal activity at least against *S. cerevisiae*. Octyl gallate exhibited a broad antimicrobial spectrum including antifungal and antibacterial activity whereas dodecyl gallate showed only antibacterial activity specifically against Gram-positive bacteria.[1,22] As far as alkyl gallates are concerned, their antimicrobial spectra and potency depend largely on the hydrophobic portion of the molecules.

The results obtained seem to provide a more rational and scientific approach to design selective and effective antibacterial agents. For example, 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,[2,19] the bactericidal activity of octyl and dodecyl gallates, especially anti-MRSA activity,[7] should be of great overall value. On the other hand, dodecyl gallate may have the potential to be used as a non-antibiotic antibacterial agent for skin treatment since *S. aureus* and *P. acnes* are important bacteria to be controlled without damaging skin.

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6. The test strains used for this study were purchased from American Type Culture Collection (Rockville, MD, USA). The procedures used for antibacterial assay were the same as previously described.[3]
7. Nonyl gallate exhibited the antibacterial activity against the six *S. aureus* strains tested, including two methicillin and two penicillin resistant strains.
8. 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 media.
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22. Antifungal octyl gallate rapidly adsorbs onto the surface of *S. cerevisiae* cells but dodecyl gallate did not.[1]