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Antibacterial Activity of Akyl Gallates against *Bacillus subtilis*

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The antibacterial activity of a series of alkyl gallates (3,4,5-trihydroxybenzoates) against Gram-positive bacteria was tested using a broth dilution method. All of the Gram-positive bacteria tested were susceptible to alkyl gallates, and this activity was found to correlate with the alkyl chain length. The antibacterial activity of alkyl gallates against *Bacillus subtilis* was a parabolic function of their lipophilicity and maximized with alkyl chain length between C_8 and C_{11}. Notably, alkyl gallates were found to be bactericidal against *B. subtilis* ATCC 9372, but this activity was significantly affected by the endospore formation in the culture. The antibacterial activity of alkyl gallates likely comes at least in part from their ability to inhibit the membrane respiratory chain but is not due to the prooxidant action.

KEYWORDS: Antibacterial activity; alkyl gallates; *Bacillus subtilis*; endospore; reactive oxygen species

INTRODUCTION

The antifungal activity of a series of alkyl gallates against *Saccharomyces cerevisiae*, *Candida albicans*, *Zygosaccharomyces bailii*, and *Aspergillus niger* was previously reported (*1*). Their antifungal activity against these fungi was due primarily to their ability to act as nonionic surface-active agents (surfactants). During this study we became aware that the same alkyl gallates are also effective against Gram-positive bacteria. Notably, these alkyl gallates exhibited bactericidal activity against *Bacillus subtilis* ATCC 9372. However, this bactericidal activity was significantly affected by the endospore formation in the culture. The preliminary data suggest that the ability of alkyl gallates to act as surfactants is unlikely to play a major role in their antibacterial activity against *B. subtilis*. Hence, their further evaluation was undertaken to gain new insights into their bactericidal action on a molecular basis.

B. subtilis is known to cause spoilage of canned vegetables, seafoods, and evaporated milk. In general, the endospore-forming species of the genera *Bacillus* is difficult to control because the endospores formed at an intracellular site are very retractile, resistant to heat, ultraviolet light, and desiccation (*2*−*4*). The aim of this paper is to describe the antibacterial activity of alkyl gallates and their structural criteria and mode of action against *B. subtilis*.

MATERIALS AND METHODS

Chemicals. A series of alkyl gallates (**1**−**11**) was available from our previous work (*5*, *6*). Gallic acid (**12**), propyl gallate (**13**), undecanol (**14**), and decanoic acid (**15**) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Gentamycin and chloramphenicol were obtained from Sigma Chemical Co. (St. Louis, MO). *N*,*N*-Dimethylformamide (DMF) was purchased from EM Science (Gibbstown, NJ). 2′,7′-Dichlorodihydrofluoresceindiacetate (DCFH-DA) was a product of Molecular Probes (Eugene, OR). Log *P* values were achieved by Chem Draw Pro version 4.5 (Cambridge Soft Co., Cambridge, MA) using Crippen's fragmentation (*7*).

Test Strains. The microorganisms, *B. subtilis* ATCC 9372, *Brevibacterium ammoniagenes* ATCC 6872, *Micrococcus luteus* ATCC 4698, *Streptococcus mutans* ATCC 25175, *Propionibacterium acnes* ATCC 11827, and *Staphylococcus aureus* ATCC 12598, were purchased from American Type Culture Collection (Manassas, VA).

Media. The NYG culture media 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 except for the case of *Strep. mutans*. For the culture of *Strep. mutans*, BHI medium consisting of 3.7% brain−heart infusion (Difco) was used. For preparation of agar plates, 1.5% agar was supplemented to the above-described media.

Preparation of Inocula. The cells of *B. subtilis* ATCC 9372 were subcloned and then kept on NYG agar plates. The cells directly taken from the plates were incubated in 20 mL of NYG broth without shaking at 37 °C for 16 h to produce the preculture. The preculture was used for the following antibacterial assay and time-kill study. For the selected compounds, preculture was also prepared using both 8- and 48-h-incubated cells.

Antibacterial Assay. Broth macrodilution methods were used as previously described (*8*). 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 NYG broth of 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 diluted culture. The assays were performed at least in triplicate on separate occasions. The final concentration of DMF in each medium was 1%, which did not affect the growth of the test bacterium.

Inactivation Study. The cultivation with alkyl 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

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the samples were spread onto NYG agar plates. After the plates had been incubated at 37 °C for 24 h, colony forming units (CFU) were estimated.

Preparation of Spores. The spores of *B. subtilis* ATCC 9372 were prepared according to the method previously described (*9*). To form spores, the cells of *B. subtilis* were grown on the NYG agar plate at 37 °C for 96 h. The spores recovered from the plate using a cell scraper were suspended into 0.9% NaCl. The spore suspension was incubated at 80 °C for 10 min to kill the rest of the viable vegetative cells prior to the spore germination test.

Measurement of Reactive Oxygen Species (ROS) Production. Cellular ROS production was examined according to a method dependent on intracellular deacylation and oxidation of DCFH-DA to the fluorescent compound 2′,7′-dichlorofluorescein as described previously (*10*). This probe was highly reactive with hydrogen peroxide and has been used in evaluating intracellular ROS generation. After preincubation of the precultivated *B. subtilis* cells (10^7 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 60 min and then washed and resuspended in 100 μL of phosphate-buffered saline. The fluorescence intensity of the cell suspension (100 μL) containing 10^7 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.

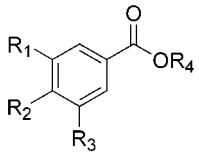
RESULTS

A series of alkyl (C_3-C_{14}) gallates (see **Figure 1** for structures) was tested for their antibacterial activity against the six selected Gram-positive bacteria, *Staph. aureus*, *Strep. mutans*, *B. subtilis*, *Brevi. ammoniagenes*, *P. acnes*, and *M. luteus*, using a broth dilution method (*8*, *11*). These bacteria all showed similar susceptibilities to alkyl gallates. Notably, as the alkyl chain length of the gallates increases, the antibacterial activity against Gram-positive bacteria did not distinctly increase. For example, octyl (C_8), nonyl (C_9), decyl (C_{10}), undecyl (C_{11}), and dodecyl (C_{12}) gallates were all found to exhibit the same MBC of 25 μg/mL against *M. luteus*. This differs from their antifungal activity described against *Sc. cerevisiae* (*6*).

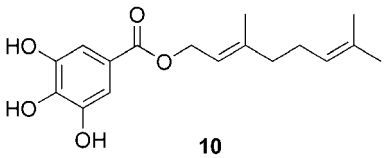
In the case against *B. subtilis*, the antibacterial activity of alkyl gallates was a parabolic function of their lipophilicity and maximized with alkyl chain length between C_8 and C_{11}. Nonyl and decyl gallates were found to be the most effective, each with an MBC of 12.5 μg/mL, followed by octyl gallate with an MBC of 25 μg/mL, as shown in **Table 1**. The activity did not disappear after the chain length reached this maximum activity. The cutoff was observed between dodecyl (C_{12}) and tridecyl (C_{13}) gallates. The differences in their MIC and MBC values were not more than 2-fold, suggesting that their activity is bactericidal. Alkyl gallates appear to inactivate the spores of *B. subtilis* as sporicides (*12*), although this was not the case. If this is so, there is notable difference between alkyl gallates and alkanols in the antibacterial activity against *B. subtilis*. Thus, alkanols were previously reported to show bacteriostatic activity against this endospore-forming bacterium and had no bactericidal activity (*8*). More specifically, tridecanol (C_{13}) was found to be the most effective among the alkanols against *B. subtilis*, with an MIC of 6.25 μg/mL, but did not exhibit any bactericidal activity up to 800 μg/mL (*8*). Similarly, neither aliphatic alkanals nor (2*E*)-alkenals exhibited any bactericidal activity against this endospore-forming bacterium up to 800 μg/mL (*13*). This is detailed more below.

In the survival experiment, the bactericidal effect of octyl gallate against *B. subtilis* was confirmed as shown in **Figure 2**. Cultures of *B. subtilis*, with a cell density of 6.5×10^5 CFU/mL, were exposed to two different concentrations of octyl gallate. The number of viable cells was determined following different periods of incubation with octyl gallate. The MIC significantly reduced the growth rate when added to the culture at the exponentially growing culture (10^6 CFU/mL), but the final cell count recovered up to 6.0×10^2 CFU/mL. No viable cells were detected after being exposed to 25 μg/mL (MBC) of octyl gallate within 2 h.

Subsequently, after the addition of 25 μg/mL of octyl gallate to the culture from early to late exponentially growth phases, the viabilities of *B. subtilis* cells were completely depleted within



1: $R_1 = R_2 = R_3 = OH$, $R_4 = CH_2(CH_2)_4CH_3$
2: $R_1 = R_2 = R_3 = OH$, $R_4 = CH_2(CH_2)_5CH_3$
3: $R_1 = R_2 = R_3 = OH$, $R_4 = CH_2(CH_2)_6CH_3$
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: $R_1 = R_2 = R_3 = OH$, $R_4 = CH_2(CH_2)_{10}CH_3$
8: $R_1 = R_2 = R_3 = OH$, $R_4 = CH_2(CH_2)_{11}CH_3$
9: $R_1 = R_2 = R_3 = OH$, $R_4 = CH_2(CH_2)_{12}CH_3$
12: $R_1 = R_2 = R_3 = OH$, $R_4 = H$
13: $R_1 = R_2 = R_3 = OH$, $R_4 = CH_2CH_2CH_3$



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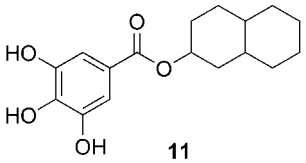

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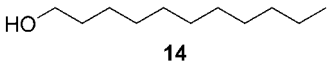

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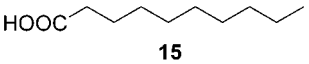

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Figure 1. Chemical structures of alkyl gallates and related compounds.

Table 1. Antibacterial Activity of Alkyl Gallates against *B. subtilis* ATCC 9372

	μg/mL		
no. of carbons	MIC	MBC	log *P*
C_3 (**13**)	800	1600	1.51
C_6 (**1**)	100	100	2.76
C_7 (**2**)	50	50	3.18
C_8 (**3**)	12.5	25	3.60
C_9 (**4**)	12.5	12.5	4.01
C_{10} (**5**)	12.5	12.5	4.43
C_{11} (**6**)	25	50	4.85
C_{12} (**7**)	25	50	5.27
C_{13} (**8**)	100	>400	5.68
C_{14} (**9**)	>400	>400	6.10
gentamycin	12.5	12.5	

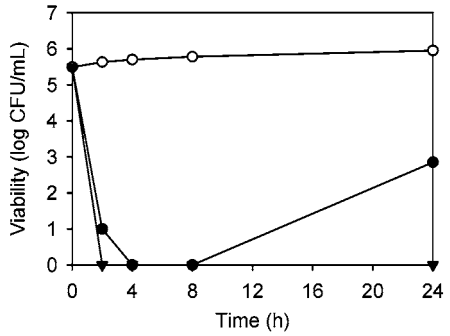

Figure 2. Bactericidal effects of octyl gallate against *B. subtilis* ATCC 9372. Exponentially growing cells of *B. subtilis* were inoculated into NYG broth and then cultured at 37 °C without shaking. Octyl gallate concentrations: 0 (○), 12.5 (●), and 25 (▼) μg/mL.

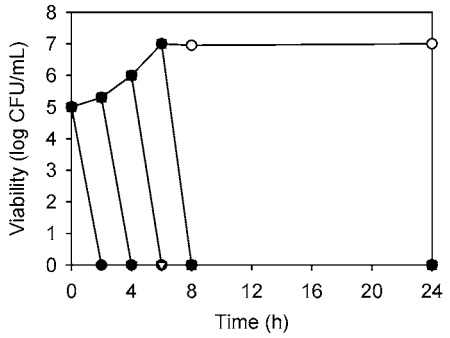

Figure 3. Effect of growth phase on the bactericidal action of octyl gallate. Cells of *B. subtilis* ATCC 9372 were incubated without shaking at 37 °C in NYG broth with added 25 μg/mL octyl gallate at 0 (●), 2 (▼), 4 (▽), and 6 (■) h. Control (○) is incubation without octyl gallate.

2 h as shown in **Figure 3**. It is evident that octyl gallate is bactericidal against *B. subtilis* from early to late exponential growth stages. Moreover, lethality was observed at cell densities of 10^5-10^7 CFU/mL, indicating that the bactericidal action of octyl gallate expresses regardless of cell density in the culture.

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 hydroxyl radicals and hydrogen peroxides in the bacteria *Salmonella typhimurium* and *Escherichia coli* (*14*). Paradoxically, alkyl gallates were previously described to trigger an apoptotic pathway in several cell lines accompanied by ROS generation. Thus, gallates were reported to induce apoptosis in human leukemia HL60 RG and to show cytotoxic effects on other cell lines (*15*−*18*). In these apoptotic processes, the generation of ROS is thought to contribute to the initiation of apoptosis (*16*, *19*, *20*). Although an estrogen mimic 4-nonylphenol induced ROS generation in the cells of *B. subtilis* dose-dependently, octyl gallate did not produce ROS, as shown in **Figure 4**. In addition, octyl gallate seemed to restrict ROS generated dose-dependently. Dodecyl gallate slightly induced ROS generation at 12.5 μg/mL, but it restricted the generation at >25 μg/mL. Hence, ROS may explain their bactericidal action. It is worthwhile to add that octyl and dodecyl gallates act rather as antioxidants and protect against oxidative damage, similar to those described (*21*).

The effects of octyl gallate against *B. subtilis* were further tested during holding viable cell number in the presence of chloramphenicol, which is known as a bacteriostatic agent that binds to the 50S ribosomal subunit and inhibits transpeptidation in protein synthesis (*22*). Octyl gallate (25 μg/mL) slightly reduced the viability of *B. subtilis* cells in the stationary phase

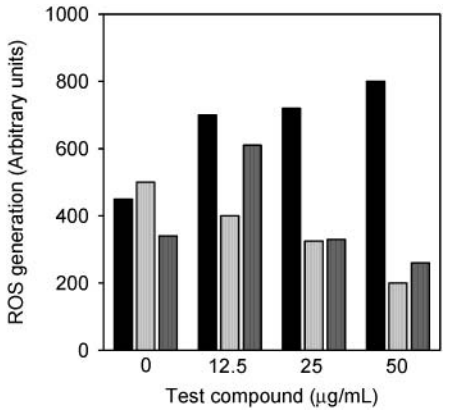

Figure 4. Effect of octyl and dodecyl gallates and *p*-nonylphenol concentrations on ROS generation in *B. subtilis* ATCC 9372 cells. After the cells had been incubated with *p*-nonylphenol (black bar), octyl gallate (light gray bar), and dodecyl gallate (dark gray bar) in NYG medium at 37 °C for 60 min, the amount of ROS generated was measured.

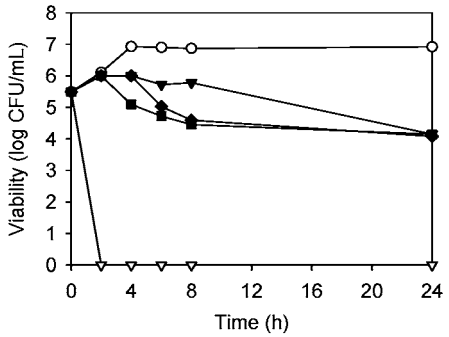

Figure 5. Effect of octyl gallate on the chloramphenicol-treated cells of *B. subtilis* ATCC 9372. Cells of *B. subtilis* were incubated without shaking at 37 °C in NYG broth with (filled symbols) and without (open symbols) 12.5 μg/mL of chloramphenicol. Octyl gallate (25 μg/mL) was added to the culture broth at 0 (triangles), 2 (rectangles), and 4 (diamonds) h.

treated with chloramphenicol as shown in **Figure 5**. The final CFU at 24 h was the same as for treatment with chloramphenicol alone. This result indicates that octyl gallate affects functions associated with cell division of *B. subtilis* such as cell wall, protein, and/or DNA biosyntheses or kills the exponentially growing cells.

The effect of nonyl gallate against the spores of *B. subtilis* was examined. The spores of *B. subtilis* were formed by 96-h cultivation and then prepared. Nonyl gallate did not kill the spores of *B. subtilis* incubated in 0.9% NaCl. Moreover, we examined its effect against spore germination of *B. subtilis*. The spores were incubated in NYG broth to germinate spores. Nonyl gallate did not affect the spore germination of *B. subtilis* (data not shown). Spores of *Bacillus* species are much more resistant than their corresponding vegetative cells to a variety of treatments including heat, radiation, and many toxic chemicals (*23*). Therefore, the bactericidal action of alkyl gallates would be weakened in the antibacterial evaluation using the culture of *B. subtilis* including spores. It is evident that nonyl gallate is not sporicidal.

The MBC values obtained were occasionally variable against the 16-h-precultivated cells of this endospore-forming bacterium. In the preculture, it is difficult to equalize completely initial inoculum size of *B. subtilis* because of the formation of endospores. Although spore formation was not observed at all after 8 h of precultivation, indicating all of the cells of *B. subtilis* are vegetatively growing cells, the formation started after 48 h

Table 2. Antibacterial Activity (Micrograms per Milliliter) of Alkyl Gallates and Related Compounds against Short- and Long-Term-Precultivated Cells of *B. subtilis* ATCC 9372

	8 h[a]		48 h[b]		
compound tested	MIC	MBC	MIC	MBC	log *P*
gallic acid (**12**)	3200	>3200	3200	>6400	0.47
octyl gallate (**3**)	25	25	25	>400	3.60
geranyl gallate (**10**)	25	25	25	400	3.45
decahydro-2-naphthyl gallate (**11**)	25	25	25	200	3.23
dodecyl gallate (**7**)	25	25	25	>400	5.27
undecanol (**14**)	25	25	25	>800	3.89
decanoic acid (**15**)	400	400	400	>800	3.27

[a] Precultivation was done at 37 °C for 8 h. [b] Precultivation was done at 37 °C for 48 h.

of precultivation. Hence, the seven selected compounds were also tested against both 8- and 48-h-precultivated cells. Thus, alkyl gallates were found to be bactericidal against *B. subtilis*, but this activity was significantly affected by the presence or absence of endospore formation in the culture. The antibacterial activity of the seven selected compounds, using the cells of *B. subtilis* after different precultivation times, was examined for comparison. As a result, octyl and dodecyl gallates did not show any bactericidal activity against 48-h-precultivated cells of *B. subtilis* as listed in **Table 2**, whereas both gallates exhibited bactericidal activity against 16-h-precultivated cells as listed in **Table 1**. It appears that the bactericidal activity of alkyl gallates against *B. subtilis* is dependent on the endospore formation in the culture. Interestingly, both geranyl gallate (**10**) and decahydro-2-naphthyl gallate (**11**) exhibited bactericidal activity even against 48-h-precultivated cells of *B. subtilis*, although to lesser extent than against 8-h-precultivated cells. This result indicates that the shape of the hydrophobic portion could affect the activity to some extent. In connection, undecanol (**14**) and decanoic acid (**15**) exhibited bactericidal activity against 8-h-precultivated cells, but neither showed any bactericidal activity against 48-h-precultivated cells of *B. subtilis* (*8*).

DISCUSSION

Alkyl gallates can be considered as head and tail structures, similar to alkanols (*8*). Therefore, the mode of their antibacterial action was expected to be as surfactants. However, it cannot be inferred from the data obtained that the antibacterial activity of alkyl gallates is the cause of the lethal effect. The antibacterial activity of alkyl gallates against *B. subtilis* was noted to be a parabolic function of their lipophilicity and maximized with alkyl chain length between C_8 and C_{11}. The length of the alkyl group is not largely associated with the potency of the activity, and this differs from their antifungal action against *Sc. cerevisiae*. In the survival experiment, lethality occurred quickly within 2 h after the addition of octyl gallate (**Figure 2**), and bactericidal activity was found at only exponentially growing and dividing cells (**Figures 3** and **4**).

Construction of a spore of *B. subtilis* initiates in response to starvation of nutrient, takes each cell ~8 h, and is directed by a tightly controlled genetic program (*24*). The MBCs of octyl and dodecyl gallates against 48-h-precultivated cells of *B. subtilis* were >400 μg/mL (**Table 2**). The long-term precultivation generates spores. Nonyl gallate did not inactivate the spores of *B. subtilis* or restrict spore germination. The spore coat is a multilayered structure surrounding the spore and composed of upward of 25 often highly cross-linked polypeptide species (*25*). Alkyl gallates could not disrupt the spore coat or pass through it to the interior of spores, indicating that these bactericidal activities are significantly affected by the endospore formation in the culture.

According to our recent findings, the antibacterial activity of alkyl gallates against Gram-positive bacteria comes in part from their ability to inhibit the membrane respiratory systems. For example, dodecyl gallate inhibited the oxygen consumption of *Pseudomonas aeruginosa* IFO 3080 cells when suspensions prepared from the same bacterial cells were incubated with dodecyl gallate. It showed dose−response for this respiratory inhibition. Dodecyl gallate also inhibited *Ps. aeruginosa* NADH oxidase by a membrane fraction prepared from the same bacterial cells (*26*). The action is not directly on ATP synthetase but earlier in the electron transport chain (ETC), similar to those found for alkanols (*27*).

The process by which alkyl gallates reach the site of action in living microorganisms must be taken into account because this process is usually neglected in the cell-free experiment. In bacteria, various enzymes, especially components of energy-converting systems such as ETCs and ATPases, are embedded in the plasma membrane, whereas in fungi they are located in the mitochondria. The ETC is a chain of specialized complex molecules (redox agents), which form a conducting path for electrons. The inner and outer surfaces of the membrane are hydrophilic, whereas the interior is hydrophobic, so the increased lipophilicity of the gallates should greatly affect their movement into the membrane lipid bilayer portions (*28*). Once inside the lipid bilayer portions, alkyl gallates may inhibit the ETC, perhaps by interfering with the redox reactions. For example, highly lipophilic dodecyl gallate can enter in part into lipid-bilayer portions and reach the ETC in bacterial membrane, but not in fungal mitochondria. This may reveal why dodecyl gallate did not show any effects on eukaryotic microorganisms such as *Sc. cerevisiae*. The pyrogallol moiety apparently plays a major role for this interference, but the length of the alkyl chain is also associated with eliciting activity to a large extent.

In the current study, the hydrolyzable ester group was selected to prevent undesired side effects, particularly the endocrine-disrupting activity of environmentally persistent estrogen mimics (*29*), such as alkylphenolic compounds (*30*). Furthermore, a series of alkyl gallates was synthesized by one-step esterification utilizing *N*,*N*′-dicyclohexylcarbodiimide (DCC) as an activating agent. Because of this synthetically easy accessibility (*11*), the construction of a wide range of structurally diverse mimics was also made available for comparison.

In summary, alkyl gallates apparently have multifunctions but biochemical mechanisms play a more essential role in their antibacterial activity against Gram-positive bacteria. Hence, the antibacterial activity of alkyl gallates against *B. subtilis* was a parabolic function of their lipophilicity and maximized with alkyl chain lengths of C_9 and C_{10}. The length of the alkyl chain is not a major contributor but is obviously significantly associated with the activity. Thus, the antimicrobial spectra and potency depend entirely on the hydrophobic portion of the molecules as far as alkyl gallates are concerned. From a practical point of view, it should also be emphasized that alkyl gallates are hydrolyzed to gallic acid and the corresponding alcohols, and both are common components in many edible plants and readily metabolized. The freed gallic acid acts as an antioxidant (*31*). The conclusion reached may provide a hint to a more rational and scientific approach to the design of effective antimicrobial agents with new modes of action.

ACKNOWLEDGMENT

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