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Antioxidant Activity of Dodecyl Gallate

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Dodecyl (C_{12}) gallate exhibits both potent chain-breaking and preventive antioxidant activity. The pyrogallol moiety is responsible for both activities. Dodecyl (lauryl) gallate prevents generation of superoxide radicals by xanthine oxidase, and this activity comes from its ability to inhibit the enzyme. The inhibition kinetics analyzed by Lineweaver−Burk plots found that dodecylgallate is a noncompetitive inhibitor for the generation of superoxide anion. Dodecyl gallate also inhibits formation of uric acid. The inhibition kinetics analyzed by Lineweaver−Burk plots found that dodecyl gallate is a competitive inhibitor for this oxidation. Mitochondrial lipid peroxidation induced by Fe(III)−adenosine 5′-diphosphate/reduced nicotinamide adenine dinucleotide was inhibited by dodecyl gallate while its parent compound, gallic acid, did not show this inhibitory activity. Dodecyl gallate protected mitochondrial functions and human red blood cells against oxidative stresses, but gallic acid showed little effect. The hydrophobic dodecyl group is largely associated with the preventive antioxidative activity.

KEYWORDS: Antioxidant activity; dodecyl gallate; gallic acid; scavenging activity; xanthine oxidase; preventive antioxidative activity

INTRODUCTION

Lipid peroxidation is well-known to be one of the reactions set into motion as a consequence of the formation of free radicals in cells and tissues. The oxidation of unsaturated fatty acids in biological membranes leads to a decrease in the membrane fluidity (*1*) and disruption of membrane structure and function (*2*, *3*). Cellular damage due to lipid peroxidation is known to associate with carcinogenesis (*4*) and other diseases (*5*). The primary biological role of antioxidants is to protect from such oxidative damage. Inhibition of membrane peroxidation has been shown to have a protective effect in the initiation and promotion of certain cancers (*6*−*8*). The past experimental studies have provided compelling evidence that antioxidants play an important role in reducing the risk of cancer. Because of the importance of discovering safe and effective antioxidants, there is considerable interest in preventive medicine in the development of antioxidants. In living systems, antioxidants may be effective in protection from oxidative damages.

During our recent study to design antibrowning agents, we became aware that the length of alkyl chain of gallates (3,4,5-trihydroxybenzoates) is significantly associated with the antioxidative activity (*9*). For example, the alkyl chain length was related to the preventive inhibition of lipid peroxidation. More specifically, cardol, 5[8(*Z*),11(*Z*),14-pentadecatrienyl]resorcinol (**1**) (see **Figure 1** for structures), was found to possess potent preventing antioxidant activity while its parent compound, resorcinol, did not show this activity. The balance of hydrophilic and hydrophobic moieties of molecules is well-known to be associated with various biological activities (*10*). However, the role of hydrophobic property of a molecule—which must be associated with biological activities—is still poorly understood. In our continuing challenge for this long-standing question, we became aware that dodecyl gallate (**2**) has been studied as a powerful radical scavenger in food but not as a preventive antioxidant. This prompted us to further study its antioxidative activity in order to gain new insights into the role of the hydrophobic alkyl group. Accumulation of this knowledge may provide a more rational and scientific approach to design safe and effective antioxidant agents. The aim of this paper is mainly to report antioxidant activity of dodecyl gallate.

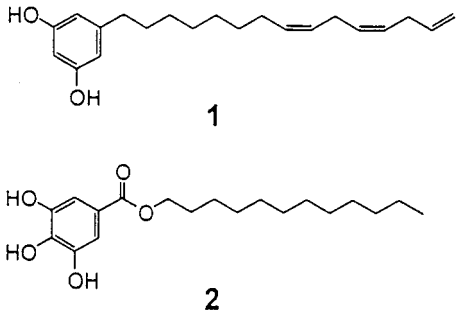

Figure 1. Chemical structures of dodecyl gallate and related compounds.

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MATERIALS AND METHODS

General Methods. General procedures were the same as previously reported (*9*). Initial velocity of the enzyme reactions was calculated from each experiment. The reaction rates were analyzed by Lineweaver−Burk and/or Dixon plots using Sigma plots.

Chemicals. Dodecyl gallate, linoleic acid, gallic acid, 1,1-diphenyl-2-*p*-picrylhydrazyl (DPPH), xanthine, 2,2′-azo-bis(2-amidinopropane) dihydrochloride (AAPH), and nitroblue tetrazolium were purchased from Aldrich Chemical Co. (Milwaukee, WI). Dodecyl gallate was recrystallized prior to the use. α-Tocopherol, cytochrome *c*, bovine serum albumin, butylated hydroxytoluene (BHT), thiobarubituric acid (TBA), and adenosine 5′-diphosphate (ADP) were obtained from Sigma Chemical Co. (St. Louis, MO). Reduced nicotinamide adenine dinucleotide (NADH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Oriental Yeast Co. (Tokyo, Japan). Succinoylated ferricytochrome *c* was prepared according to the method previously described (*11*).

Assay of Autoxidation. Oxidation of linoleic acid was measured as previously described (*12*). The test samples dissolved in 120 μL of ethanol were added to a reaction mixture in a screw cap vial. Each reaction mixture consisted of 2.28 mL of 2.51% linoleic acid in ethanol and 9 mL of 40 mM phosphate buffer (pH 7.0). The vial was placed in an oven at 40 °C. At intervals during incubation, a 0.1 mL aliquot of the mixture was diluted with 9.7 mL of 75% ethanol, which was followed by adding 0.1 mM of 30% ammonium thiocyanate. Precisely 3 min after the addition of 0.1 mL of 20 mM ferrous chloride in 3.5% hydrochloric acid to the reaction mixture, the absorbance at 500 nm was measured.

Radical Scavenging Activity on DPPH. First, 1 mL of 100 mM acetate buffer (pH 5.5), 1.87 mL of ethanol, and 0.1 mL of ethanolic solution of 3 mM DPPH were put into a test tube. Then, 0.03 mL of the sample solution (dissolved in dimethyl sulfoxide (DMSO) was added to the tube and incubated at 25 °C for 20 min. The absorbance at 517 nm (DPPH, $\epsilon = 8.32 \times 10^3$) 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.

Assay of Lipoxygenase. The soybean lipoxygenase 1 (EC 1.13.11.12, Type I) used for the bioassay was purchased from Sigma Chemical Co. The reaction mixture consisted of 1.2 mM linoleic acid, 60 mM phosphate buffer (pH 7.0), and different concentration of sample. At zero time, the enzyme solution (100 units) was added to the reaction mixture. The lipoxygenase activity was measured polarographically (*13*) with an OBH 100 oxygen electrode at 25 °C.

Assay of Superoxide Anion Generated by Xanthine Oxidase. The xanthine oxidase (EC 1.1.3.22, Grade IV) used for the bioassay was purchased from Sigma Chemical Co. Superoxide anion was generated enzymatically by xanthine oxidase system. The reaction mixture consisted of 2.70 mL of 40 mM sodium carbonate buffer containing 0.1 mM ethylenediaminetetraacetic acid (EDTA; pH 10.0), 0.06 mL of 10 mM xanthine, 0.03 mL of 0.5% bovine serum albumin, 0.03 mL of 2.5 mM nitroblue tetrazolium, and 0.06 mL of sample solution (dissolved in DMSO). To the mixture at 25 °C, 0.12 mL of xanthine oxidase (0.04 units) was added, and the absorbance at 560 nm was recorded for 90 s (by formation of blue formazan) (*14*). The control experiment was carried out by replacing sample solution with the same amount of DMSO.

Assay of Uric Acid Formation Generated by Xanthine Oxidase. The reaction mixture consisted of 2.76 mL of 40 mM sodium carbonate buffer containing 0.1 mM EDTA (pH 10.0), 0.06 mL of 10 mM xanthine, and 0.06 mL of sample solution (dissolved in DMSO). The reaction was started by the addition of 0.12 mL of xanthine oxidase (0.04 Unit), and the absorbance at 293 nm was recorded for 90 s. In the case of dodecyl gallate, the concentration of DMSO was increased to 8% because of the low solubility of dodecyl gallate in the reaction mixture.

Preparation of Mitochondria and Microsomes. Livers of Wistar male rats weighing 100−150 g were removed quickly and dropped into ice-cold 3 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose and 0.1 mM EDTA. Mitochondria were obtained as a pellet after centrifugation at 15 000*g* and then resuspended in 100 mM *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (HEPES) buffer (pH 7.2). Submitochondrial particles were prepared by sonication (*15*) of mitochondrial suspension for 1 min at 4 °C using a model 450 Sonifier (Branson Ultrasonics Co., U.S.A.). Microsomes were obtained as a pellet after centrifugation at 105 000*g* for 60 min (*16*), and then, the pellet was resuspended in the buffer containing 70 mM sucrose, 0.21 M mannitol, 0.1 mM EDTA, and 3 mM HEPES (pH 7.4). Protein concentrations of the suspensions were determined by the method of Lowry et al. (*17*).

Measurement of Lipid Peroxidation. The NADPH-dependent peroxidation of microsomal lipid was assayed by the modified method described by Pederson et al. (*18*). Rat liver microsomes (equivalent 0.2 mg protein) were incubated at 37 °C in 1 mL of reaction mixture containing 0.05 M Tris-HCl buffer (pH 7.5), 2 mM ADP, 0.12 mM $Fe(NO_3)_3$, and 0.1 mM NADPH. The reaction was initiated by the addition of NADPH. After 5 min, 2 mL of TCA−TBA−HCl reagent (15% w/v trichloroacetic acid; 0.375% TBA; 0.25 N HCl) and 90 μL of 2% BHT were added to the reaction mixture. The solution was heated for 15 min in a boiling water bath. After it was cooled, the flocculent precipitate was removed by centrifugation at 1000*g* for 10 min. The absorbance of TBA reactive substances in the supernatant was determined at 535 nm (*19*).

Mitochondrial lipid peroxidation was assayed by the modified method described by Takayanagi et al. (*20*). Rat liver submitochondrial particles (equivalent 0.3 mg of protein) were incubated at 37 °C in 1 mL of reaction mixture containing 50 mM HEPES buffer (pH 7.0), 2 mM ADP, 0.1 mM $FeCl_3$, 10 μM rotenone, and 0.1 mM NADH. The reaction was initiated by the addition of NADH. After 5 min, the reaction was terminated and lipid peroxidation was determined by the same TBA method as for the microsomal peroxidation.

Assay of Superoxide Anion Generated in Microsomes. Rat liver microsomes were diluted to a concentration of 0.4 mg/mL with 0.1 M Tris-HCl buffer containing 0.1 mM EDTA and 30 μM succinoylated cytochrome *c*. The reaction mixture was incubated at 37 °C for 30 s. NADPH was added to the final concentration of 0.2 mM. The reduction of succinoylated cytochrome *c* was monitored using the absorbance at wavelength pair 550 minus 557 nm (*11*). The O_2^- -mediated reduction of cytochrome *c* was discriminately detected by the addition of an excess of superoxide dismutse (purchased from Sigma Chemical Co., EC 1.15.1.1) that suppresses the direct enzymatic reduction of cytochrome *c*.

Preparation of Erythrocyte and Assay for Hemolysis. Blood from healthy donors was collected in heparinized tubes. Erythrocytes were separated, by centrifugation, from plasma and buffy coat and were washed three times with saline. During the last washing, the cells were centrifuged at 2000*g* for 10 min to obtain a constantly packed cell preparation. A 10% suspension of erythrocytes in the solution containing 152 mM NaCl and 10 mM sodium phosphate buffer (pH 7.4) was preincubated at 37 °C for 5 min before addition of same volume of 100 mM AAPH in the same buffer saline. The reaction mixture was gently shaken at 37 °C. At intervals during incubation, 2 aliquots were taken out from the mixture, the one aliquot was diluted with 20 volumes of 0.15 M NaCl, and the solution was centrifuged at 1000*g* for 10 min. The absorbance of the supernatant (*A*) was determined at 540 nm. The other aliquot was distilled with 20 volumes of water to yield complete hemolysis and centrifuged. The absorbance of the supernatant (*B*) was determined at 540 nm. The percent hemolysis was calculated from the ratio of the absorbance (A/B) × 100 according to the equation previously described (*21*).

RESULTS

To clarify how the hydrophobic moiety contributes to antioxidant activity, dodecyl gallate was selected for the present study as a model, because this particular gallate is one of the three gallates that are currently permitted to use as antioxidant additives in food (*22*). The activity of dodecyl gallate was compared with that of its parent compound, gallic acid, that lacks the hydrophobic alkyl moiety. Both are known to act as radical scavengers (*22*, *23*). It should be noted that the highest

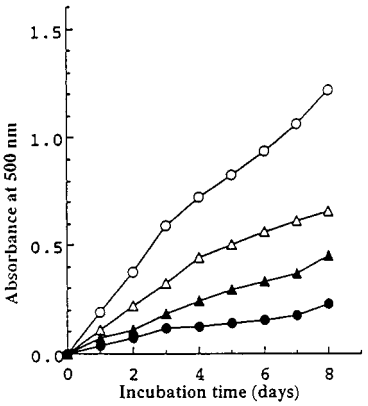

Figure 2. Antioxidative activity of dodecyl gallate and gallic acid. Each compound was added at a final concentration of 30 μg/mL based on the total volume of solution. ●, Dodecyl gallate; ▼, gallic acid; ▽, α-tocopherol; and ○, control.

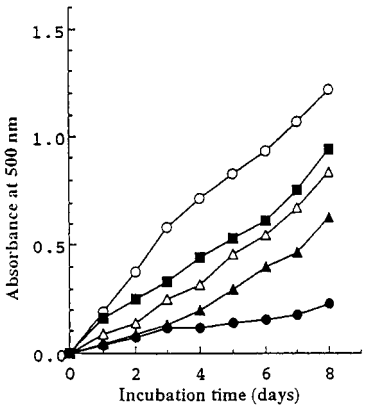

Figure 3. Concentration effect of antioxidative activity of dodecyl gallate. The symbols show the final concentration of dodecyl gallate added to the total reaction volume. ●, 30; ▲, 10; △, 3; ■, 1; and ○, 0 μg/mL. The rate indicated is an average rate of an experiment (three determinations).

concentration tested throughout this antioxidant experiment was 30 μg/mL unless otherwise specified, because of the solubility limitation of dodecyl gallate in water-based test solutions. Therefore, discussions are on the basis of data with only significant activity.

Membrane lipids are known to contain relatively large amounts of unsaturated fatty acids. Lipid peroxidation is a typical free radical oxidation and proceeds via a cyclic chain reaction (*24*). Linoleic acid is especially the target of lipid peroxidation. Effect of gallic acid and dodecyl gallate on autoxidation of linoleic acid was measured by the ferric thiocyanate method as previously described (*12*). The result is shown in **Figure 2**. In a control reaction, the production of lipid peroxide increased almost linearly during 8 days of incubation. α-Tocopherol, also known as vitamin E, inhibited the linoleic acid peroxidation almost 50% at 30 μg/mL. In preventing this oxidation, both gallic acid and dodecyl gallate were found to be more effective than α-tocopherol, and dodecyl gallate was more effective as compared to gallic acid. The result indicates that dodecyl group is not essential but related to the activity to some extent. **Figure 3** shows the dose−response of dodecyl gallate. The activity of dodecyl gallate was found to be potent, and about 50% inhibition was still observed at 10 μg/mL. Both gallic acid and dodecyl gallate have the ability to donate a hydrogen atom to the peroxy radical derived from the autoxidizing fatty acids and hence act as radical scavengers.

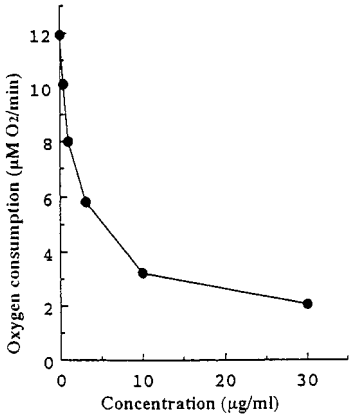

Figure 4. Effect of dodecyl gallate on soybean lipoxygenase. The rate reported is an average rate of an experiment (three determinations).

The human body produces free radicals during the course of its normal metabolism. Free radicals are even required for several normal biochemical processes. For example, the phagocyte cells involved in the body's natural immune defenses generate free radicals in the process of destroying microbial pathogens. If free radicals are produced during the normal cellular metabolism in sufficient amounts to overcome the normally efficient protective mechanisms, metabolic and cellular disturbances will occur in a variety of ways. Evidence is accumulating that several cell types other than phagocytes also produce extracellular free radicals in vivo. For example, lipids are oxidized by lipoxygenases and cyclooxygenases, which generate peroxide intermediates. More specifically, lipoxygenases catalyze the oxygenation of polyenoic fatty acids containing a 1(*Z*),4(*Z*)-pentadiene system such as linoleic acid and arachidonic acid to their 1-hydroperoxy-2(*E*),4(*Z*)-pentadiene product. In this connection, lipoxygenases are of importance since they may generate peroxides in human low-density lipoproteins (LDL) in vivo and facilitate the development of arteriosclerosis, a process in which lipid peroxidation appears to be intimately involved (*25*, *26*). Soybean lipoxygenase was used if dodecyl gallate inhibited the linoleic acid peroxidation by this enzyme. The result is shown in **Figure 4**. Dodecyl gallate inhibited the linoleic acid peroxidation 80% at 30 μg/mL (88.8 μM), and IC_{50} was established as 3.5 μg/mL (10.3 μM).

In addition, xanthine oxidase produces superoxide anion ($O_2\cdot^-$) as a normal product (*27*). The one electron reduction products of O_2, superoxide anion ($O_2\cdot^-$), hydrogen peroxide (H_2O_2), and hydroxy radical ($HO\cdot$) from $O_2\cdot^-$ participate in the initiation of lipid peroxidation (*28*), resulting in tissue injury (*29*). The presence of these unpaired electrons causes the species to be highly reactive. Because of the high reactivity, free radicals are able to produce metabolic disturbances and to damage membrane structures in a variety of ways. The effect of dodecyl gallate on the generation of superoxide anion by xanthine oxidase was tested, and the result is shown in **Figure 5**. Dodecyl gallate was effective to inhibit the generation of superoxide anion catalyzed by xanthine oxidase. Thus, 90% inhibition was observed at 68 μg/mL (200 μM) and 70% inhibition at 19.6 μg/mL (56 μM), and IC_{50} was established as 12.8 μg/mL (38 μM). Kinetic analysis of dodecyl gallate analyzed by Lineweaver−Burk plots is shown in **Figure 6**. The result shows that dodecyl gallate is a noncompetitive inhibitor to the enzyme. It can decrease the apparent value of V_m with no effect on that of K_m. The kinetic constants obtained from the experimental data were recorded in **Table 1**. This behavior is observed that dodecyl gallate can combine with both free enzyme and the

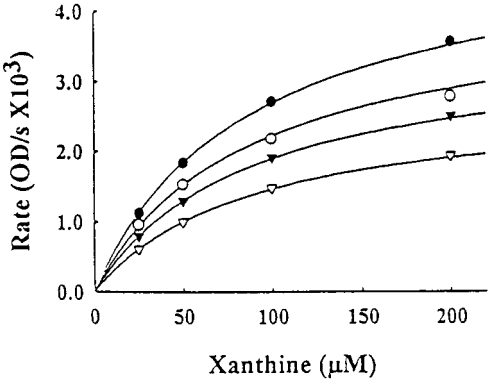

Figure 5. Generation of superoxide anion by xanthine oxidase in the presence of dodecyl gallate. Concentrations of dodecyl gallate are 0 (●), 10 (○), 20 (▼), and 40 (▽) μM, respectively. The rate indicated is an average rate of an experiment (three determinations).

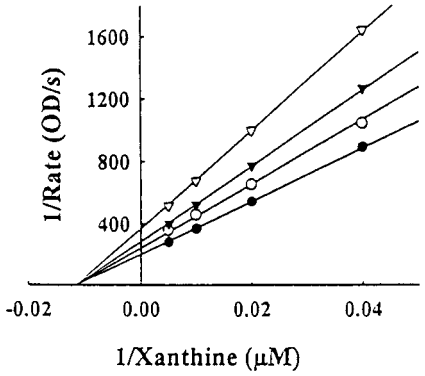

Figure 6. Lineweaver–Burk plots of superoxide anion generation by xanthine oxidase in the presence of dodecyl gallate. Concentrations of dodecyl gallate are 0 (●), 10 (○), 20 (▼), and 40 (▽) μM, respectively.

Table 1. Inhibition Constants of Gallic Acid, Dodecyl Gallate, and α-Tocopherol for Superoxide Anion Formation by Xanthine Oxidase and DPPH Scavenging Activity

constants	gallic acid	dodecyl gallate	α-tocopherol
IC_{50}	6.7 ± 1.0 μM[b]	38 ± 2 μM	220 ± 20 μM
K_m	88 ± 5 μM	86 ± 8 μM	87 ± 10 μM
V_m	4.53×10^{-3} OD/s	5.01×10^{-3} OD/s	4.53×10^{-3} OD/s
inhibition	reversible	reversible	reversible
inhibition type	competitive	noncompetitive	noncompetitive
K_I	2.8 ± 0.2 μM	47 ± 3 μM	220 ± 14 μM
DPPH scavenging[b]	6.51[a] ± 0.30	7.28 ± 0.54	2.48 ± 0.11

[a] Mean ± SD. [b] Scavenged DPPH molecules per an inhibitor's molecule.

enzyme−substrate complex to inhibit generation of superoxide anion. Gallic acid also inhibited this enzyme as a competitive inhibitor, indicating that dodecyl group may not be related to this inhibitory activity but the pyrogallol moiety plays a role in direct modulation. It appears most likely that superoxide anion was generated and scavenged by these two pyrogallol derivatives. However, this does not eliminate the possibility that dodecyl gallate inhibits, at least in part, the enzyme-catalyzed reaction by inhibiting the enzyme. In addition, α-tocopherol also inhibits the enzyme-catalyzed reaction as a noncompetitive inhibitor. The equilibrium constant for inhibitor binding, K_I, was obtained as 2.8 μM for gallic acid. This is approximately 17- and 78-fold more potent than those of dodecyl gallate and α-tocopherol, respectively.

Xanthine oxidase, a molybdenum-containing enzyme, converts xanthine to uric acid. This enzyme-catalyzed reaction is known to proceed via transfer of an oxygen atom to xanthine from the molybdenum center. Hence, formation of uric acid was measured. Dodecyl gallate inhibits this oxygen atom transfer reaction as a competitive inhibitor as shown in **Figure 7**, and IC_{50} was established as 100 μM. The kinetic and inhibition constants of dodecyl gallate are listed in **Table 2**. The equilibrium constant for inhibitor binding, K_I, was obtained as 55.5 μM. Interestingly, gallic acid did not inhibit xanthine oxidase up to 100 μM, indicating that the dodecyl group plays an important role in eliciting the activity. It appears that the antioxidant activity of dodecyl gallate is not only due to radical scavenging but also due to inhibiting the enzyme. In brief, dodecyl gallate has multiple functions, though the sharp distinction may not be appropriate. In this connection, α-tocopherol did not inhibit the enzyme up to 100 μM.

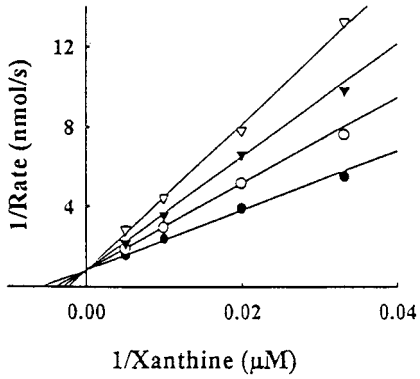

Figure 7. Lineweaver–Burk plots of uric acid generation by xanthine oxidase in the presence of dodecyl gallate. Concentrations of dodecyl gallate are 0 (●), 25 (○), 50 (▼), and 80 (▽) μM, respectively.

Table 2. Inhibition Constants of Dodecyl Gallate for Xanthine Oxidase

IC_{50}	100 ± 4 μM[a]
K_m	178 ± 26 μM
V_m	1.20 ± 0.20 nmol/s
inhibition	reversible
inhibition type	competitive
K_I	55.5 ± 5.2 μM

[a] Mean ± SD (from three experiments).

Biomembranes, such as microsomes, erythrocytes, and the plasma membrane, are rich in polyunsaturated fatty acids, which are sensitive to peroxidative damage induced by oxygen free radicals. Membrane lipids are particularly susceptible to oxidation not only because of their high polyunsaturated fatty acid content but also because of their association in the cell membrane with enzymic and nonenzymic systems capable of generating free radical species (*30*, *31*). Microsomes, especially smooth-surfaced endoplasmic reticulum, easily produce lipid peroxides and are thought to supply the peroxidation products to other tissues (*32*). NADPH−P-450 reductase and cytochrome P-450 are involved in NADPH-induced microsomal lipid peroxidation (*33*, *34*). Lipid peroxidation occurs when rat liver microsomes are incubated with Fe(III)−ADP/NADPH (*19*), and this can be measured by the TBA method, since the major products of microsomal lipid peroxidation are the aldehydic compounds such as malondialdehyde and 4-hydroxyalk-2-enals. Dodecyl gallate completely inhibited microsomal NADPH−P-450 reductase-dependent lipid peroxidation at 30 μg/mL (**Figure 8**). This inhibitory activity of dodecyl gallate was still observed about 83% and even as low as at 10 μg/mL. However, its parent compound, gallic acid, exhibited little effect on this enzymatically induced lipid peroxidation.

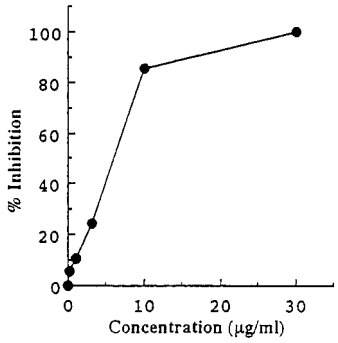

Figure 8. Inhibitory effect of dodecyl gallate on NADPH-dependent microsomal lipid peroxidation.

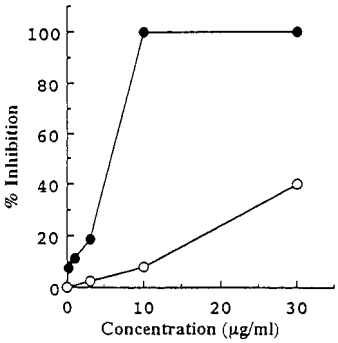

Figure 9. Inhibitory effect of dodecyl gallate (●) and gallic acid (○) on NADH-dependent mitochondrial lipid peroxidation.

Because redox reactions are most prominent in mitochondria, they are constantly susceptible to oxidative stress (*35*, *36*). In mitochondrial electron transport system, at least two sites of the mitochondrial electron transport system leak and react with oxygen to generate $O_2{\cdot}^-$ and, subsequently, H_2O_2 (*37*). Lipid peroxides, produced by HO·, derived from $O_2{\cdot}^-$ and H_2O_2, affect mitochondrial function (*38*). Lipid peroxidation by submitochondrial particles is supported by NADH or NADPH in the presence of ADP and Fe(III) (*20*). As shown in **Figure 9**, dodecyl gallate completely inhibited mitochondrial lipid peroxidation at 10 μg/mL while gallic acid inhibited less than 10% at this concentration.

The cellular sources of $O_2{\cdot}^-$ in mammalian cells include the microsomal electron-transfer chain, entering a slow electron transfer to O_2 via NADPH−cytochrome P-450 reductase. In microsomes, cytochrome *c* is reduced by the normal electron transport and also by superoxide anion. To avoid the normal reduction of cytochrome *c* by microsomal flavoprotein NADPH−cytochrome P-450 reductase, succinoylated cytochrome *c* was used. The rate of $O_2{\cdot}^-$ was determined by measuring the reduction rate of succinoylated cytochrome *c* using NADPH as a substrate. As shown in **Figure 10**, dodecyl gallate inhibited this reduction. The result indicates that dodecyl gallate is effective in preventing the generation of $O_2{\cdot}^-$ in rat liver microsome.

The lipids in the red cell membrane are highly unsaturated, and the red cells are exposed to a higher oxygen tension than any other tissues. Furthermore, the red cells are packed with hemoglobin, one of the most powerful catalysts capable of initiating lipid peroxidation (*39*). When human erythrocytes were incubated in air at 37 °C, they were stable and little hemolysis occurred within 5 h. The peroxy radicals generated by thermal decomposition of an azo-initiator AAPH induce the free radical chain oxidation in erythrocyte membrane (*21*). During 5 h incubation with AAPH, almost 100% of erythrocytes was damaged. Dodecyl gallate inhibited the lysis of human red cells

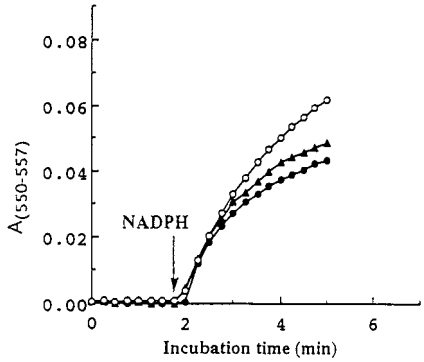

Figure 10. Effect of dodecyl gallate on generation of superoxide anion in microsomes. ●, 10; ▲, 1 μg/mL; and ○, control.

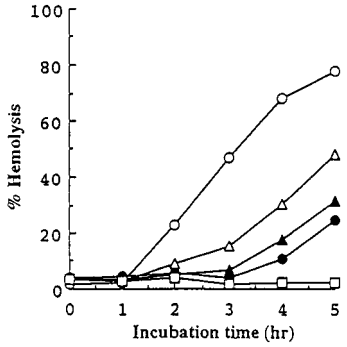

Figure 11. Inhibition of oxidative haemolysis by dodcyl gallate. ●, 10; ▲, 3; △, 1; ○, 0 μg/mL; and □, without AAPH.

due to the peroxy radical attack as shown in **Figure 11**. Almost 100% inhibition was observed at 10 μg/mL.

It appears that dodecyl gallate inhibits autoxidation (**Figures 2** and **3**) and enzymic peroxidation (**Figure 4**) of linoleic acid and prevents the generation of superoxide radicals by xanthine oxidase (**Figures 5** and **6**). In living systems, it also inhibits lipid peroxidation (**Figures 8** and **9**), generation of free radical species (**Figures 10**), and protects the membrane damage from reactive oxygen species (**Figure 11**). The dodecyl group is evidently associated with most of these antioxidant activities. It is conceivable that dodecyl gallate prevents lipid peroxidation of membrane in cells and organelles from oxidative stress.

DISCUSSION

Oxidative degradation of polyunsaturated fatty acids occurs in two sequential steps of initiation and propagation (*33*). Therefore, antioxidative materials acting in living systems are classified as preventive antioxidants and chain-breaking ones (*40*). Gallic acid scavenged superoxide anion generated by xanthine oxidase, which would be grouped as a chain-breaking antioxidant. On the other hand, dodecyl gallate strongly inhibited lipid peroxidation in microsomes and mitochondria, which would be classified as a preventive one. There are significant differences in the effectiveness of gallic acid and dodecyl gallate as antioxidants. The latter compound's antioxidant activity is not only due to radical scavenging but also due to preventing. There is no doubt that their antioxidant activity is due primarily to the pyrogallol moiety in the molecules, although gallic acid and its alkyl chain esters, regardless of their carbon chain length, showed potent scavenging activity on the DPPH radical (*23*). In brief, inhibition of the enzyme increases with increasing the alkyl chain length. It appears that the addition of alkyl group to gallic acid introduced the preventive antioxidant activity. The

hydrophobic dodecyl group very likely plays a role in direct modulation, but the precise explanation for this still remains unclear.

The site of antioxidant localization is important. It is not clear, however, if this gallate is delivered, without being metabolized, to the sites where antioxidants are needed in living systems. For instance, xanthine oxidase occurs almost exclusively in the liver and small intestinal mucosa in mammals and, more importantly, this oxidase exists in the cells. The lipophilic dodecyl group, which is associated with the preventive antioxidant activity of dodecyl gallate, may allow to partition into lipophilic membranes of cells and organelles, where this ester presumably exerts its antioxidant activity in the protection from oxidative damage, similar to the phytyl chain (*41*) in tocopherols and tocotrienols (*42*). In connection with this, ascorbyl palmitate can be considered as another similar example (*43*). It is worthwhile to add that introducing a dodecyl group to gallic acid is likely adding surface-active property. As a result, dodecyl gallate is amphiphilic but gallic acid is not.

Safety is a primary consideration for antioxidants that may be utilized in unregulated quantities. After it is consumed, dodecyl gallate is likely hydrolyzed, at least in part, to gallic acid and dodecyl alcohol, which are common constituents in many edible plants. It is worthwhile to note that gallic acid may act as a potent antioxidant; for example, it scavenges superoxide anion generated by xanthine oxidase in living systems but does not possess the preventive antioxidant activity observed with dodecyl gallate. On the other hand, dodecyl alcohol likely prevents generation of superoxide anion and hydrogen peroxide by mitochondria in the resting state as an uncoupler, similar to fatty acids (*44*). It is clear that the hydrophobic dodecyl group is related to elicit the antioxidative activity. Interestingly, dodecyl alcohol was previously described to exhibit antibacterial activity (*45*) but not antioxidant activity. In contrast, gallic acid is well-known to possess antioxidant activity (*22*) but not antibacterial activity. We now found that dodecyl gallate shows antibacterial activity, in addition to the potent antioxidant activity described above (*23*). It appears that pyrogallol moiety is the primary response to elicit the antioxidant activity; hence, adding this moiety to other molecules can be considered as introducing this additional activity. However, it needs to be borne in mind that the hydrophobic portion is also related to the activity, although its precise role is still obscure. It needs to be borne in mind that dodecyl gallate is an ester that can be hydrolyzed after consumption.

Last, it should be kept in mind that antioxidants may exert prooxidant effects on other molecules in living system (*22*), although dodecyl gallate is currently permitted for use as an antioxidant additive in food. Antioxidant status is the balance between the antioxidant system and peroxidants in living organisms. This balance is dynamic and, in the human body, is probably tipped slightly in favor of oxidation, which is essential for the production of energy (*26*). It seems that the antioxidants needed are those that do not destroy this balance. The results hitherto obtained indicate that the biological significance of dodecyl gallate and other antioxidants need to be fully evaluated not only from one aspect but also from a whole and dynamic perspective. The fact that dodecyl gallate is currently permitted for use as an antioxidant in food should be of considerable advantage for further evaluations. Accumulation of this knowledge may provide a more rational and scientific approach to design and select safe and effective antioxidant agents. For example, pyrogallol derivatives such as 5-alk(en)ylpyrogallols and similar compounds may be worthwhile to evaluate their antioxidant activity. On the basis of this concept, we tested several phenolic compounds previously isolated from edible plants and found cardols, 5-alk(en)ylresorcinols, to possess potent preventive antioxidant activity. In addition, dodecyl gallate was recently noted as a nonantibiotic antibacterial agent (*46*).

LITERATURE CITED

(1) Dobrestova, G. E.; Borschevskaya, T. A.; Petrov, V. A.; Vladimirov, Y. A. The increase of phospholipid bilayer rigidity after lipid peroxidation. *FEBS Lett*. **1977**, *84*, 125−128.

(2) Machlin, L.; Bendich, A. Free radical tissue damage: protective role of antioxidant nutrients. *FASEB J*. **1987**, *1*, 441−445.

(3) Slater, T. F.; Cheeseman, K. H. Free radical mechanisms in relation to tissue injury. *Proc. Nat. Soc*. **1987**, *46*, 1−12.

(4) Yagi, K. Lipid peroxides and human disease. *Chem. Phys. Lipid* **1987**, *45*, 337−341.

(5) Fuller, C. J.; Jialal, I. Antioxidants and LDL oxidation. In *Antioxidants and Disease Prevention*; Garewal, H. S., Ed.; CRC Press: Boca Raton, FL, 1997; pp 115−130.

(6) McLarty, J. W. Antioxidants and cancer: The epidemiologic evidence. In *Antioxidants and Disease Prevention*; Garewal, H. S., Ed.; CRC Press: Boca Raton, FL, 1997; pp 45−65.

(7) Mayne, S. T.; Ziegler, R. G. Antioxidant nutrients and lung cancer. In *Antioxidants and Disease Prevention*; Garewal, H. S., Ed.; CRC Press: Boca Raton, FL, 1997; pp 67−86.

(8) Garewal, H. S. Antioxidant nutrients and oral cavity cancer. In *Antioxidants and Disease Prevention*; Garewal, H. S., Ed.; CRC Press: Boca Raton, FL, 1997; pp 87−95.

(9) Kubo, I.; Kinst-Hori, I.; Kubo, Y.; Yamagiwa, Y.; Kamikawa, T.; Haraguchi, H. Molecular design of antibrowning agents. *J. Agric. Food Chem*. **2000**, *48*, 1393−1399.

(10) Hansch, C.; Dunn, W. J., III. Linear relationships between lipophilic character and biological activity of drugs. *J. Pharm. Sci*. **1972**, *61*, 1−19.

(11) Kuthan H.; Ulrich, V. A quantitative test for superoxide radicals produced in biological systems. *Biochem. J*. **1982**, *203*, 551−558.

(12) Osawa, T.; Namiki, M. A novel type of antioxidant isolated from leaf wax of *Eucalyptus* leaves. *Agric. Biol. Chem*. **1981**, *45*, 735−739.

(13) Kristie, D.; Thompson, J. E. Inhibition of lipoxygenase activity − A cautionary note. *Phytochemistry* **1989**, *28*, 2577−2581.

(14) Toda, S.; Kumura, M.; Ohnishi, M. Effects of phenolcarboxylic acids on superoxide anion. *Planta Med*. **1991**, *57*, 8−10.

(15) Takeshige, K.; Minakami, S. NADH- and NADPH-dependent formation of superoxide anions by bovine heart submitochondrial particles and NADH-ubiquinone reductase preparation. *Biochem. J*. **1979**, *180*, 129−135.

(16) Liu, G.; Zhang, T.; Wang, B.; Wang, Y. Protective action of seven natural phenolic compounds against peroxidative damage to biomembranes. *Biochem. Pharmacol*. **1992**, *43*, 147−152.

(17) Lowry, O. H.; Rosebrough, H. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem*. **1951**, *193*, 265−275.

(18) Pederson, T. C.; Buege, J. A.; Aust, S. D. Microsomal electron transport. *J. Biol. Chem*. **1973**, *248*, 7134−7141.

(19) Buege, J. A.; Aust, S. D. Microsomal lipid peroxidation. In *Methods in Enzymology*; Fleischer, S., Packer, L., Eds.; Academic Press: New York, 1978; Vol. 52, pp 302−310.

(20) Takayanagi, R.; Takeshige, K. Minakami, S. NADH- and NADPH-dependent lipid peroxidation in bovine heart submitochondrial particles. Dependence on the rate of electron flow in the respiratory chain and antioxidant role of ubiquinol. *Biochem. J*. **1980**, *192*, 853−860.

(21) Miki, M.; Tamai, H.; Mino, N.; Yamamoto, Y.; Niki, E. Free-radical chain oxidation of rat red blood cells by molecular oxygen and its inhibition by α-tocopherol. *Arch. Biochem. Biophys*. **1987**, *258*, 373−380.

(22) Aruoma, O. I.; Murcia, A.; Butler, J.; Halliwell, B. Evaluation of the antioxidant and prooxidant actions of gallic acid and its derivatives. *J. Agric. Food Chem.* **1993**, *41*, 1880−1885.
(23) Kubo, I. Molecular design of antioxidative and antimicrobial agents. *Chemtech* **1999**, *29* (8), 37−42.
(24) Witting, L. A. Vitamin E and lipid antioxidants in free-radical-initiated reactions. In *Free Radicals in Biology*; Pryor, W. A., Ed.; Academic Press: New York, 1980; Vol. 4, pp 295−319.
(25) Kubow, S. Lipid oxidation products in food and atherogenesis. *Nutr. Rev.* **1993**, *51*, 33−40.
(26) Davies, K. J. Oxidative stress: the paradox of aerobic life. *Biochem. Soc. Symp.* **1995**, *61*, 1−31.
(27) Fong, K. L.; McCay, P. B.; Poyer, J. L.; Steele, B. B.; Misra, H. Evidence that peroxidation of lysosomal membranes is initiated by hydroxy free radicals produced during flavin enzyme activity. *J. Biol. Chem.* **1973**, *248*, 7792−7797.
(28) Comporti, M. Lipid peroxidation. An overview. In *Free Radicals: From Basic Science to Medicine*; Poli, G., Albamo, E., Dianzani, M. U., Eds; Birkhäuser Verlag: Switzerland, 1993; pp 65−79.
(29) Mayumi, T.; Schiller, H. J.; Bulkley, G. B. Pharmaceutical intervention for the prevention of post-ischemic reperfusion injury. In *Free Radical: From Basic Science to Medicine*; Poli, G., Albano, E., Dianzni, M. U., Eds.; Birkhäuser Verlag: Switzerland, 1993; pp 438−457.
(30) Sevanian, A.; Hochstein, P. Mechanism and consequences of lipid peroxidation in biological systems. *Annu. Rev. Nutr.* **1985**, *5*, 365−390.
(31) Halliwell, B.; Gutteridge, J. M. C. Role of free radicals and catalytic metal ions in human disease: an overview. In *Methods in Enzymology*; Packer, L., Glazer, A. N., Eds.; Academic Press: New York, 1990; Vol. 186, pp 1−85.
(32) Roders, M. K. NADPH-dependent microsomal lipid peroxidation and the problem of pathological action at a distance: new data on induction of red cell damage. *Biochem. Pharmacol.* **1978**, *27*, 437−443.
(33) Svingen, B. A.; Buege, J. A.; O'Neal, F. O.; Aust, S. D. The mechanism of NADPH-dependent lipid peroxidation. The propagation of lipid peroxidation. *J. Biol. Chem.*, **1979**, *245*, 5892−5899.
(34) Ekstrom, G.; Ingerman-Sundberg, M. Rat liver microsomal NADPH-supported oxidase activity and lipid peroxidation dependent on ethanol-inducible cytochrome P-450 (P-450 IIEI). *Biochem. Pharmacol.* **1989**, *38*, 1313−1319.
(35) Hruszkewycz, A. Evidence for mitochondrial DNA damage by lipid peroxidation. *Biochem. Biophys. Res. Commun.* **1988**, *153*, 191−197.
(36) Wiswedel, I.; Ulbright, O.; Augustin, W. Studies of lipid peroxidation in isolated rat heart mitochondria. *Biomed. Biochim. Acta* **1989**, *2*, 73−76.
(37) Nohl, H. Oxygen radical release in mitochondria: Influence of age. In *Free Radical, Aging, and Degenerative Diseases;* Johnson, J. E., Ed.; Alan R. Liss Inc.: New York, 1986; pp 77−97.
(38) Forman, H. J.; Boveris, A. Superoxide radical and hydrogen peroxide in mitochondria. In *Free Radicals in Biology*; Pryor, W. A., Ed.; Academic Press: New York, 1982; Vol. 5, pp 65−90.
(39) Chiu, D.; Lubin, B.; Shohet, S. B. Peroxidative reactions in red cell biology. In *Free Radicals in Biology*; Pryor, W. A., Ed.; Academic Press: New York, 1982; Vol. 5, pp 115−160.
(40) Halliwell, B.; Gutteridge, J. M. C. The antioxidants of human extracellular fluids. *Arch. Biochem. Biophys.* **1990**, *280*, 1−8.
(41) Burton, G. W.; Joyce, A.; Ingold, K. U. Is vitamin E the only lipid-soluble, chain-breaking antioxidant in human blood plasma and erythrocyte membranes? *Arch. Biochem. Biophys.* **1983**, *221*, 281−290.
(42) Papas, A. M. Vitamin E: Tocopherols and tocotrienols. In *Antioxidant Status, Diet, Nutrition, and Health*; Papas, A. M., Ed.; CRC Press: Boca Raton, FL, 1999; pp 189−230.
(43) Cort, W. M. Antioxidant activity of tocopherols, ascorbyl palmitate, and ascorbic acid and their mode of action. *J. Am. Oil Chem. Soc.* **1974**, *51*, 321−325.
(44) Korshunov, S. S.; Korkina, O. V.; Ruuge, E. K.; Skulachev, V. P.; Starkov, A. A. Fatty acids as natural uncouplers preventing generation of $O_2^{\bullet -}$ and H_2O_2 by mitochondria in the resting state. *FEBS Lett.* **1998**, *435*, 215−218.
(45) Kubo, I.; Muroi, H.; Kubo, A. Structural functions of antimicrobial long-chain alcohols and phenolics. *Bioorg. Med. Chem.* **1995**, *3*, 873−880.
(46) Kubo, I.; Xiao, P.; Fujita, K. Antifungal activity of octyl gallate: Structural criteria and mode of action. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 347−350.

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