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Effects of alkyl chain length of gallate on self-association and membrane-binding

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Running title: self-association and membrane-binding of alkyl gallates

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Summary

Alkyl gallates are anticipated for their use as antibacterial and antiviral agents. Although their pharmacological activities depend on their alkyl chain length, no mechanism has yet been clarified. As described herein, we investigated the membrane binding properties of a series of alkyl gallates using fluorescence measurement to elucidate their different pharmacological activities. Membrane binding of the alkyl gallates increased concomitantly with increasing alkyl chain length, except for cetyl gallate and stearyl gallate. Dynamic light scattering revealed that alkyl gallates with a long alkyl chain are prone to self-association in the solution. Membrane binding abilities of the alkyl gallates are correlated with antibacterial and antiviral activities, as described in previous reports. The partition constants of the alkyl gallates to lipid membranes depend on the membrane components and the membrane phase. Self-association and lipid binding of the alkyl gallates might be primary biophysical factors associated with their pharmacological activities.

Keywords: Alkyl gallate; Lipid membrane; Self-association; Partition constant; Solubility

Abbreviations: **C1**, methyl gallate; **C2**, ethyl gallate; **C3**, propyl gallate; **C4**, butyl gallate; **C8**, octyl gallate; **C12**, dodecyl gallate; **C16**, cetyl gallate; **C18**, stearyl gallate; Chol, cholesterol; DLS, dynamic light scattering; DOPC, dioleoylphosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; DPPC, dipalmitoylphosphatidylcholine

1. Introduction

Gallate is a general term used to describe salts and esters of gallic acid with the galloyl group. Some gallate compounds, typically catechin and proanthocyanidin gallates, have been developed for pharmacological applications [1–7]. Among them, alkyl gallates have recently been regarded as affecting microbial cell viability [8–18], virus activity [19–25], and human leukemia cell proliferation [26]. The alkyl gallates' pharmacological activity increases concomitantly with their alkyl chain length. Therefore their activity has been attributed to their surfactant-like effects, which can induce lipid membrane disruption and membrane protein inactivation [11–13]. In fact, alkyl gallates have both a hydrophilic galloyl group and hydrophobic alkyl chain. More hydrophobic species with longer alkyl chains, e.g. dodecyl gallate [8,9] and stearyl gallate [10,11], do not function as antibacterial agents, suggesting an unknown factor that determines the cutoff point for activity [9].

Fluorescence spectra have been used often for analyses of interactions of various aromatic compounds with lipid membranes because fluorescence spectra depend greatly on the solution environment [27]. For example, membrane binding of peptides and low-molecular-weight compounds has been observed through changes in fluorescence intensity [28–34]. Furthermore, fluorescence analyses present the advantage of being quantitative. For that reason, the thermodynamic parameters of the interactions, such as partition constants, can be determined. The

membrane binding affinity for catechin derivatives has been measured to investigate their pharmacological activities [35].

Although partition constants of alkyl gallates with lipid membranes might be related to their pharmacological activity, partition constants have not been examined aside from qualitative observations [36]. This study revealed that the partition constant depends on the alkyl chain length of gallate. The constant of the alkyl gallates increased concomitantly with increasing hydrophobicity, except for longer alkyl chain species such as cetyl and stearyl gallates. These two alkyl gallates are exceptional probably because of their self-association detected by dynamic light scattering measurements. Moreover, the membrane-binding of alkyl gallates was found to be correlated with the pharmacological activity of a series of alkyl gallates. Consequently, our findings are useful for understanding not only the physicochemical properties of alkyl gallates, but also for elucidating their pharmacological activity.

2. Materials and methods

Chemicals: All alkyl gallates were obtained from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). Dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), and cholesterol were obtained from NOF Corp. (Tokyo, Japan) along with sodium dihydrogen phosphate (Nacalai Tesque Inc., Kyoto, Japan) and ethanol (Kanto Chemical Co. Inc., Tokyo, Japan). All compounds were of the highest commercially available grade.

2.1 Solubility measurement of alkyl gallates: The respective solubilities of alkyl gallates in 10 mM phosphate buffer (pH 7.4) were measured as follows [38]. An appropriate amount of alkyl gallate powder was transferred into a test tube, to which 1.5 ml of 10 mM phosphate buffer (pH 7.4) was added. The suspension was heated at 40 °C for 1 h with frequent vortexing for complete dissolution of alkyl gallate powders. The solution was then incubated at 25 °C for 3 days with frequent vortexing. Subsequently, the suspension was centrifuged at 25 °C and $16,000 \times g$ for 20 min to obtain a supernatant saturated with the alkyl gallates. After appropriate dilution of the supernatant with water, the supernatant absorbance was determined spectrophotometrically at 271 nm using a UV–VIS spectrophotometer (ND-1000; NanoDrop Technologies, Inc., Wilmington, DE, USA). The absorbance value was converted to the concentration based on the standard curve determined for

methyl gallate. Solubility was determined in triplicate, from which the averages and standard errors were obtained.

2.2 Observation of self-association of alkyl gallates: To verify the self-association of the alkyl gallates, dynamic light scattering (DLS) measurements of them at 3 μM were conducted at 25 $^{\circ}\text{C}$ using a light-scattering spectrometer (DLS-7000; Ostuka Electronics Co., Ltd. Osaka, Japan) equipped with an argon ion laser at scattering angles of 90° [46]. Samples that did not pass the instrument's internal quality criteria were omitted.

2.3 Preparation of lipid membranes: Large unilamellar vesicles (LUV) composed of DOPC, DOPG, DPPC, and cholesterol (Chol) were used as model lipid membranes, prepared using the extrusion method with 200 nm pore size polycarbonate membranes. The appropriate amounts of 5 mM lipids were mixed in chloroform. Subsequently, the solvent was removed completely in a vacuum desiccator connected to a rotary vacuum pump for 12 h. To this dry lipid film, 1 ml of 10 mM phosphate buffer (pH 7.4) was added; then the 1.5 mM lipid suspension was vortexed for several seconds above the phase-transition temperature (25 $^{\circ}\text{C}$ for DOPC and DOPG, and 40 $^{\circ}\text{C}$ for DPPC). Subsequently, the solutions were extruded through 200-nm-pore-size polycarbonate membranes

(Avanti Mini-Extruder; Avanti Polar Lipids, Inc., Alabaster, AL) at a temperature higher than the phase-transition temperature.

2.4 Fluorescence analysis of the binding of alkyl gallates to phospholipid membranes: Fluorescence spectra of 3 μM alkyl gallate in 10 μM lipids with 10 mM phosphate buffer (pH 7.4) were recorded at 25 °C. Only for **C8–18** were they presolubilized in ethanol before mixing with the lipid solution to determine the alkyl gallate concentration. Thereby, the final component of the solution was 3 μM alkyl gallate, 10 μM lipids, 0.6% ethanol, and 10 mM phosphate buffer (pH 7.4), where the residual ethanol did not affect their fluorescence spectra. Finally, the fluorescence intensity of each reference solution without the alkyl gallate was subtracted from the intensity of the sample solutions. The determined intensities were averages of triplicate experiments. Thereby, the average and standard error were obtained. These spectra were measured using a spectrofluorometer (FP6500; Jasco Corp., Tokyo, Japan). The emission fluorescence spectra were recorded for 200–500 nm, using the excitation wavelength of 271 nm.

2.5 Measurement of the partition constant of alkyl gallates to the membranes: To determine the partition constant of the alkyl gallates (**C8–C16**) to the membranes, the changes in fluorescence intensity of the alkyl gallate on the membrane-binding were monitored using a spectrofluorometer.

The prepared sample solutions contained various concentrations of the lipids comprising DOPC, DPPC/DOPC or DPPC/Chol, 60 nM alkyl gallates, 0.6% ethanol, and 10 mM phosphate buffer (pH 7.4). The fluorescence intensity of each reference solution without the alkyl gallates was subtracted from the intensity of the sample solutions. The determined intensities were averages of triplicate experiments: the average and standard error were obtained.

3. Results

3.1 Solubility and hydrodynamic radius of alkyl gallates: The alkyl gallates used for this study are shown in Scheme 1, where they are termed **C1–C18** according to the carbon number of the alkyl chain. The solubility of the alkyl gallates in 10 mM phosphate buffer solution was measured for physicochemical characterization of the alkyl gallates. As shown in Table 1, the solubility of **C2** is higher than that of **C1**, which is consistent with our previous study [37, 38]. The solubility of **C12** is lower than that of **C16**, which is not surprising when considering the dynamic light scattering measurements as follows. Results show that **C16** and **C18** tend to self-associate in the solution (Fig. 1). The solutions of **C16** and **C18** respectively exhibit a unimodal size distribution with mean diameters of 84.6 ± 4.6 nm and 130.6 ± 17.6 nm. In contrast, **C8** and **C12** were not detected using the same apparatus. It can be concluded that the self-association of **C16** and **C18** increases their apparent solubilities.

3.2 Binding of alkyl gallates to phospholipid membranes: Binding of the alkyl gallates to the membranes was observed through the change of fluorescent intensities of their aromatic moiety. Figure 2 presents fluorescence spectra of **C4** in the presence or absence of DOPG or DOPC. As expected, the fluorescence spectrum of **C4** differed in the presence of positively charged DOPG or amphoteric DOPC. Accordingly, results suggest that the binding of **C4** to the membranes is mainly

attributable to the hydrophobic interaction between DOPC and the alkyl chains of the alkyl gallates. Similar data were obtained for **C1**, **C2**, and **C3** (Fig. S1). To support the suggestion presented above, we investigated the dependence of the alkyl chain length of the binding to DOPC. Ratios of the fluorescence intensity in the presence of DOPC to that in the absence of DOPC increased with elongation of the alkyl chain length (Fig. 3A). The ratios for **C1–C12** increased with the alkyl chain length (Fig. 3, see Fig. S2 for their spectral data). The changes in fluorescence intensities of their aromatic moiety are caused by the polarity of the solution environment [31]. In contrast, the fluorescence intensity of **C16**, despite its longer alkyl chain, was weaker than that of **C12**. Additionally, the intensity of **C18** did not change according to the presence of the lipid membrane.

3.3 Estimation of the partition constant of alkyl gallates to phospholipid membranes: Partition constants of the alkyl gallates to the membranes were estimated from the binding isotherms as follows. Figure 4A shows representative data for the peak intensity of the fluorescence spectra of **C8** in various concentrations of DOPC, as normalized by the maximum value. The normalized intensity in Fig. 4A increased by the addition of DOPC and subsequently reached a plateau at which all **C8** molecules bind to the membrane. Here, a two-state transition was presumed: binding or non-binding states of the alkyl gallates to the membrane. Additionally, it was assumed that the alkyl gallates bind to both the internal and external monolayer. Consequently, the equilibrium concentration of the alkyl

gallate in the bulk solutions, as denoted by C_{eq} , was obtained as [28]

$$C_{eq} = C_T - C_M = C_T(1 - F_N), \quad (1)$$

where C_T and C_M respectively denote the total concentration of the alkyl gallate in solution and its concentration in the lipid membranes. Therein, F_N denotes the normalized fluorescence intensity corresponding to the vertical axis in Fig. 4A. The molar ratio of the alkyl gallate molecules in the membranes to total lipid molecules, denoted by X_b , is given as

$$X_b = C_T F_N / C_L, \quad (2)$$

where C_L is the lipid concentration in solution. Finally, because the ratio of the concentration of the alkyl gallate to that of the lipid membrane in the solution is low, X_b can be expressed as [29]

$$X_b = K_{int} C_{eq}, \quad (3)$$

where K_{int} is the intrinsic partition constant of the alkyl gallate to the lipid membrane. According to these analyses, X_b and C_{eq} are actually mutually proportional (Fig. 4B). These calculations are

applicable to the other alkyl gallates, except for **C1–C4** and **C18**: because of their weak binding affinity to the membrane, their binding curves were not obtained. The partition constants for **C8**, **C12** and **C16** are presented in Table 2; the partition constants for **C12** and **C16** were of the same order and an order of magnitude higher than that for **C8**. These results reflect that the partition constant for **C16** might be affected by self-association of **C16**, as described above (Fig. 1).

3.4 Effect of membrane components on the binding of alkyl gallate to phospholipid membranes: The membrane liquid–solid phase differs according to its components [39]. They can be expected to affect the binding affinity of the alkyl gallates. Figure 5A exhibits the partition constants for **C8**, **C12**, and **C16** to the lipid membranes composed of various ratios of DPPC/DOPC. The partition constants for these alkyl gallates increased concomitantly with the increasing ratio of DOPC to DPPC; the value for DOPC was twice that for DPPC. Figure 5B shows alteration of the partition constants for these alkyl gallates to the membranes containing cholesterol. The partition constants for **C8** and **C12** decreased concomitantly with increasing concentration of cholesterol, although that for **C16** was maintained.

4. Discussion

Alkyl gallates are anticipated for use as antibacterial and antiviral agents because various studies have already shown their pharmacological activity in vitro [8–25]. Therefore, their biophysical properties should be clarified in advance of their application. Nevertheless, quantitative studies such as thermodynamic studies of the binding interaction of the alkyl gallates to lipid membranes have not been reported. Additionally, it has not been established why highly hydrophobic alkyl gallates with a longer alkyl chain did not function as antibacterial agents [8–11]. Regarding the binding interaction of the alkyl gallates to lipid membranes, only qualitative observations have been performed [36]. In this study, we quantitatively examined the binding ability of alkyl gallates with different alkyl chain lengths to various lipid membranes. It is worth mentioning that this is the first report of a partition constant between the alkyl gallates and phospholipid membrane. Ratios of fluorescence intensity of **C1–C12** have increased concomitantly with increasing alkyl chain length (Fig. 3). The ratios for **C1–C12** increased with the logarithm of the octanol–water partition coefficient ($\log P$) of the alkyl gallates. Therefore, it was suggested that the membrane-binding ability of **C1–C12** depends on their hydrophobicity derived from the alkyl chain. However, for more hydrophobic species with a longer alkyl chain (**C16, C18**), the intensity decreased. Here, recall that DLS measurements for **C8–C18** indicated self-association of **C16** and **C18**, even at concentrations below solubility (Fig. 1, Table 1). Assuming that the self-associated forms have no ability to interact

with the lipid membrane, the observed reduction of the membrane-binding ability for **C16** and **C18** can be ascribed to the promotion of self-association with increasing alkyl chain length. The result, that **C16** but not **C18** interacted with the membranes (Fig. 3B), presents the possibility that the self-association state of **C16** is an equilibrium, with the soluble monomeric state binding to the lipid membranes.

Reportedly, antibacterial and antiviral activities of the alkyl gallates decrease as their alkyl chain length increases beyond a certain length [8–11], indicating a cutoff point for the activity of the alkyl gallates [9]. Reduction of the activity will be reasonable according to our observations. The reduction can be attributed to the promotion of the self-association with increasing alkyl chain length. In other words, such self-associated species can not interact with the bacterial cell membranes or envelopes of viruses. Therefore, their activities would be reduced. Consequently, the pharmacological activity of the alkyl gallates can be expected to be associated closely with their physicochemical properties such as hydrophobicity and the monomeric solubility. To assess the relation between the physicochemical properties and the pharmacological activity, we investigated the correlation between the membrane binding ability and pharmacological activity (Table 3), where the binding ability was evaluated using the ratios of the fluorescence intensity of the alkyl gallates (F/F_0). Most correlation coefficients were positive and greater than 0.7. Therefore, the membrane-binding ability of the alkyl gallates will probably account for the results obtained for the

pharmacological activity. It is noteworthy that the correlation coefficients for *Trichophyton rubrum* and *Microsporum gypseum* were lower than the others. Consequently, the pharmacological activity of the alkyl gallates might be partly accounted for by other functions including disruption of lipid membranes, inactivation of membrane proteins and their downstream activities.

The alkyl gallates' interaction with cholesterol and with phospholipids also constitutes important information. Lipid membranes composed of DPPC, DOPC, and cholesterol are often used as cell membrane models with phase changes depending on the composition ratio [39–44]. The results presented in Fig. 5 indicate that the alkyl gallates are more stable in the liquid phase of DPPC/DOPC than in the solid phase, although they are less stable in the liquid phase of DPPC/Chol than in the solid phase. Consequently, the stability of alkyl gallates in lipid membranes depends substantially on the membrane components rather than the membrane phase. The direct interaction of the alkyl gallates with the lipid components might be related with various pharmacological effects toward bacterial cells [12,14] and viruses [19,21].

Finally, the stability of the alkyl gallate in aqueous solution depends on the co-existing solutes, as shown in our previous study [37,38]. Alteration of the stability in the solution by the solutes can affect the partition constants and thereby affect their activity toward bacterial cells and viruses. Such effects of solutes are potentially applicable to prevent self-association and to enhance their

bioavailability as drugs and food additives. We are carrying out systematic investigations of the solute effects to elucidate their potential benefits.

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Table 1. Solubility of the alkyl gallates in 10 mM phosphate buffer (pH 7.4) at 25 °C

Gallate	Solubility (M)
Metyl (C1)	$6.46 \pm 0.11 \times 10^{-2}$
Ethyl (C2)	$8.19 \pm 0.05 \times 10^{-2}$
Propyl (C3)	$1.92 \pm 0.01 \times 10^{-2}$
Butyl (C4)	$1.21 \pm 0.02 \times 10^{-2}$
Octyl (C8)	$5.7 \pm 0.6 \times 10^{-4}$
Dodecyl (C12)	$6.4 \pm 0.8 \times 10^{-5}$
Cetyl (C16)	$9.1 \pm 0.7 \times 10^{-5}$
Stearyl (C18)	$3.9 \pm 0.7 \times 10^{-5}$

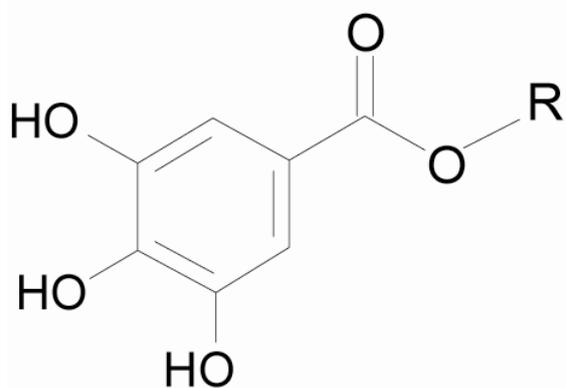
Table 2. Intrinsic partition constant (K_{int}) of octyl (**C8**), dodecyl (**C12**) and cetyl (**C16**) gallates to the lipid membrane of DOPC

Gallate	K_{int} (1/M)
Octyl (C8)	$1.5 \pm 0.1 \times 10^5$
Dodecyl (C12)	$8.2 \pm 0.7 \times 10^5$
Cetyl (C16)	$8.4 \pm 0.6 \times 10^5$

Table 3. Calculated correlation coefficients between ratios of the fluorescence intensity (F/F_0) and the pharmacological activity of the alkyl gallates against bacteria and viruses

Targets	$r^{[a]}$	Ref.
<i>Propionibacterium acnes</i>	0.949	[13]
<i>Brevibacterium ammoniagenes</i>	0.892	[13]
<i>Staphylococcus aureus (MRSA)</i>	0.892	[13]
<i>Micrococcus luteus</i>	0.824	[13]
<i>Bacillus subtilis</i>	0.824	[13]
<i>Streptococcus mutans</i>	0.593	[13]
<i>Trichophyton mentagrophytes</i>	0.409	[16]
<i>Trichophyton rubrum</i>	0.135	[16]
<i>Microsporium gypseum</i>	0.129	[16]
Targets	$r^{[b]}$	Ref
<i>Herpes simplex virus type-1</i>	0.745	[19]
<i>Lenzites betulina</i>	0.785	[14]
<i>Gloeophyllum trabeum</i>	0.729	[14]
<i>Chaetomium globosum</i>	0.716	[14]
<i>Trametes versicolor</i>	0.683	[14]

[a]: Correlation coefficients between ratios of the fluorescence intensity (F/F_0) determined in this paper and antibacterial activities of the alkyl gallates ($\log(1/\text{MIC})$), where MIC is the minimum inhibitory concentration, as described in reports of previous studies [13,16]. [b]: Correlation coefficients between F/F_0 and the yield of antiviral effects [19] and antifungal fractions [14]. The calculated correlation coefficients are the Pearson product-moment correlation coefficients [45].



- C 1** R = CH₃
C 2 R = CH₂CH₃
C 3 R = (CH₂)₂CH₃
C 4 R = (CH₂)₃CH₃
C 8 R = (CH₂)₇CH₃
C12 R = (CH₂)₁₁CH₃
C16 R = (CH₂)₁₅CH₃
C18 R = (CH₂)₁₇CH₃

Scheme 1. Chemical structures of the alkyl gallates.

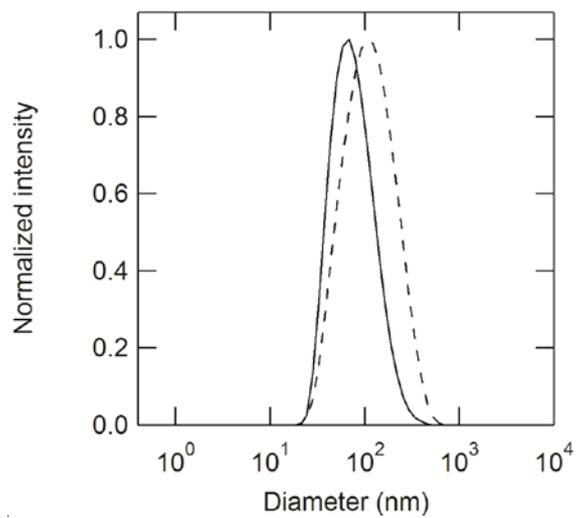


Figure 1. DLS measurements for 3 μM cetyl gallate (**C16**) (solid line) and stearyl gallate (**C18**) (broken line) in water.

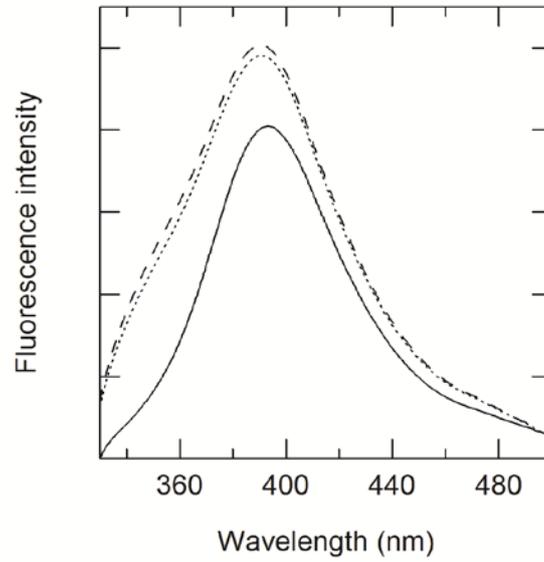


Figure 2. Fluorescence spectra of 3 μM butyl gallate (**C4**) in the presence and absence of 10 μM DOPC or DOPG: no lipid, solid line; DOPC, broken line; DOPG, dotted line. The excitation wavelength of the fluorescence spectra is 271 nm.

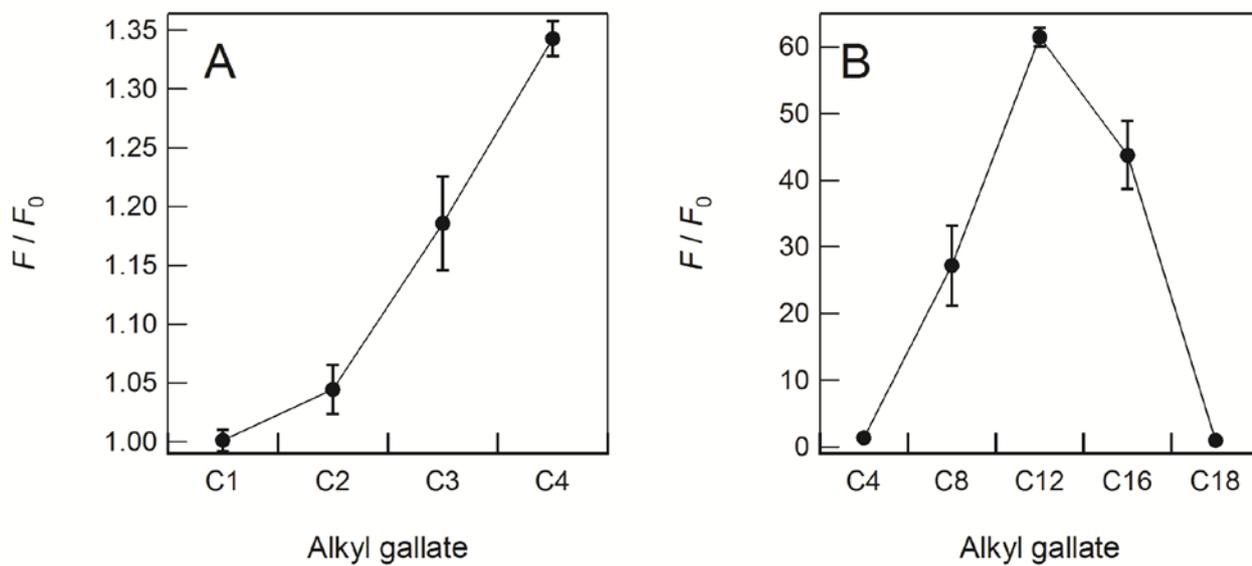


Figure 3. Ratios of the fluorescence intensity of the respective 3 μM alkyl gallates (A) C1–C4 and (B) C4–C18 in the presence of 10 μM DOPC to those in the buffer solution.

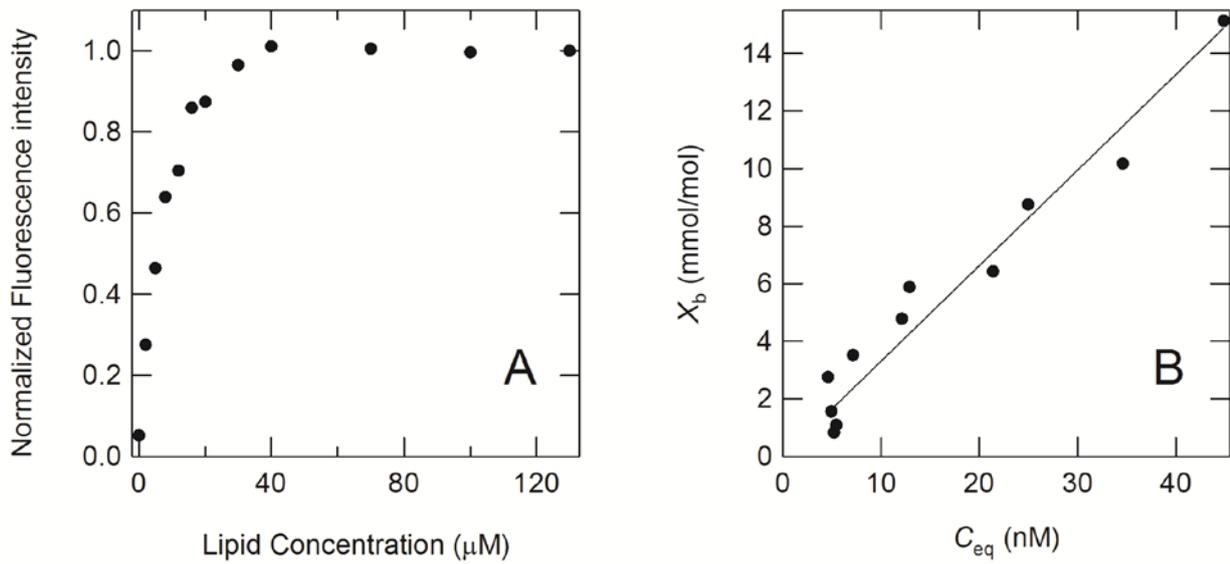


Figure 4. (A) Normalized fluorescent intensity of 60 nM octyl gallate (**C8**) at 350 nm in various concentrations of DOPC, normalized by the maximum value. (B) Binding isotherms of octyl gallate (**C8**) to the lipid membrane of DOPC at 25 °C.

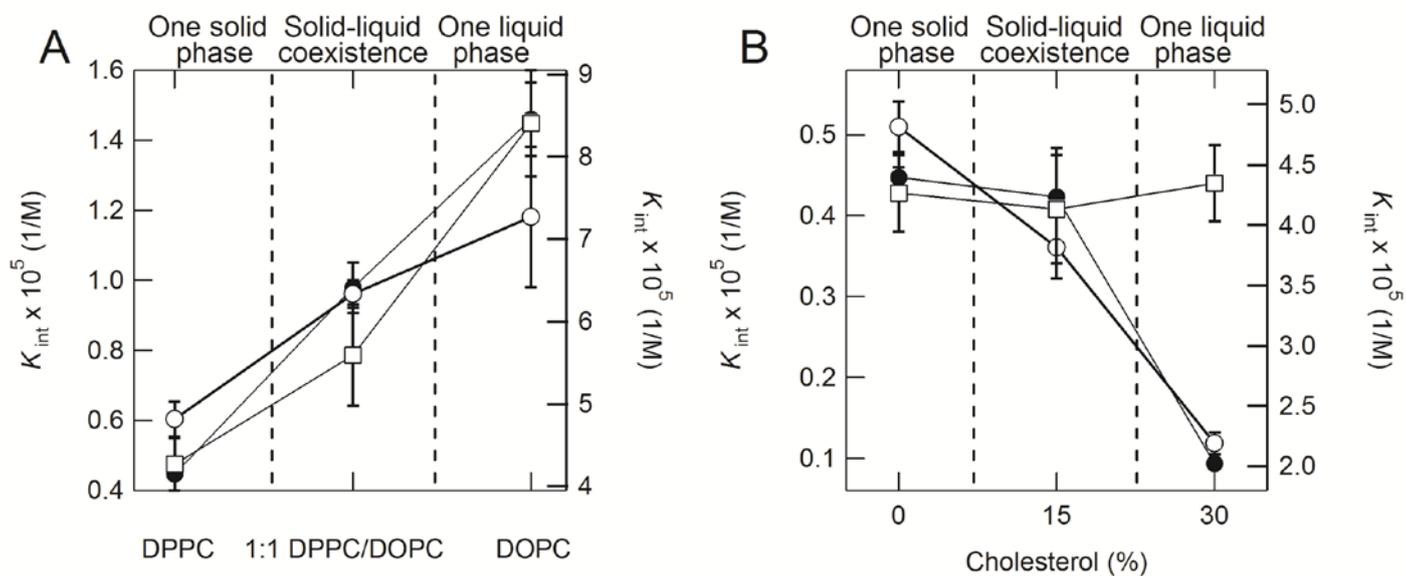


Figure 5. Binding constants (K_{int}) of 60 nM octyl gallate (C8) (closed circles, left axes), dodecyl gallate (C12) (open circles, right axes) and cetyl gallate (C16) (open squares, right axes) in the presence of DPPC/DOPC (A) and DPPC/Chol (B).

Supporting Information

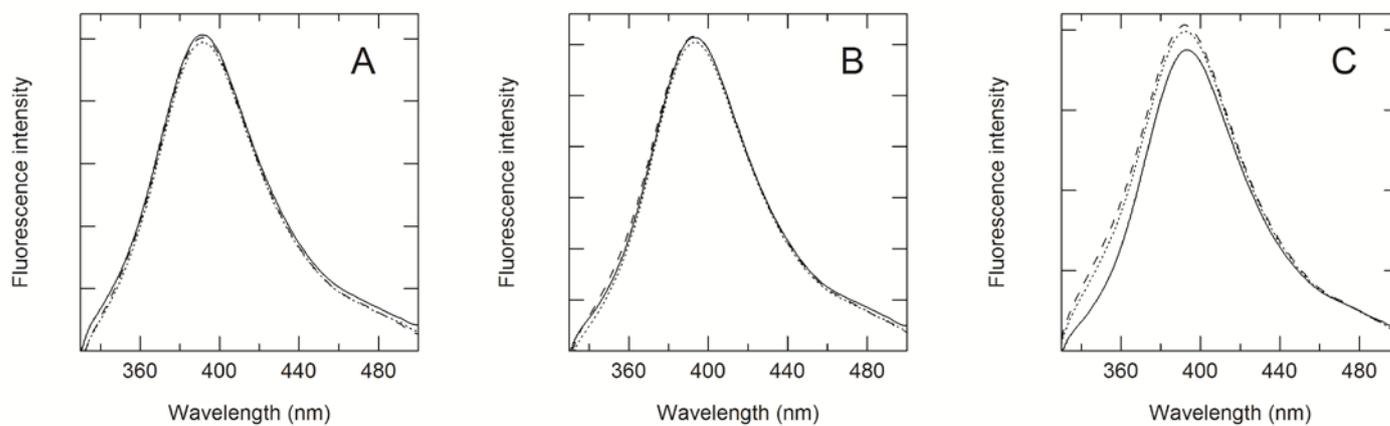


Figure S1. Fluorescence spectra of 3 μM methyl gallate (**C1**) (A), ethyl gallate (**C2**) (B) and propyl gallate (**C3**) (C) in the presence or absence of 10 μM DOPC or DOPG: no lipid, solid line; DOPC, broken line; DOPG, dotted line. The excitation wavelength of the fluorescence spectra is 271 nm.

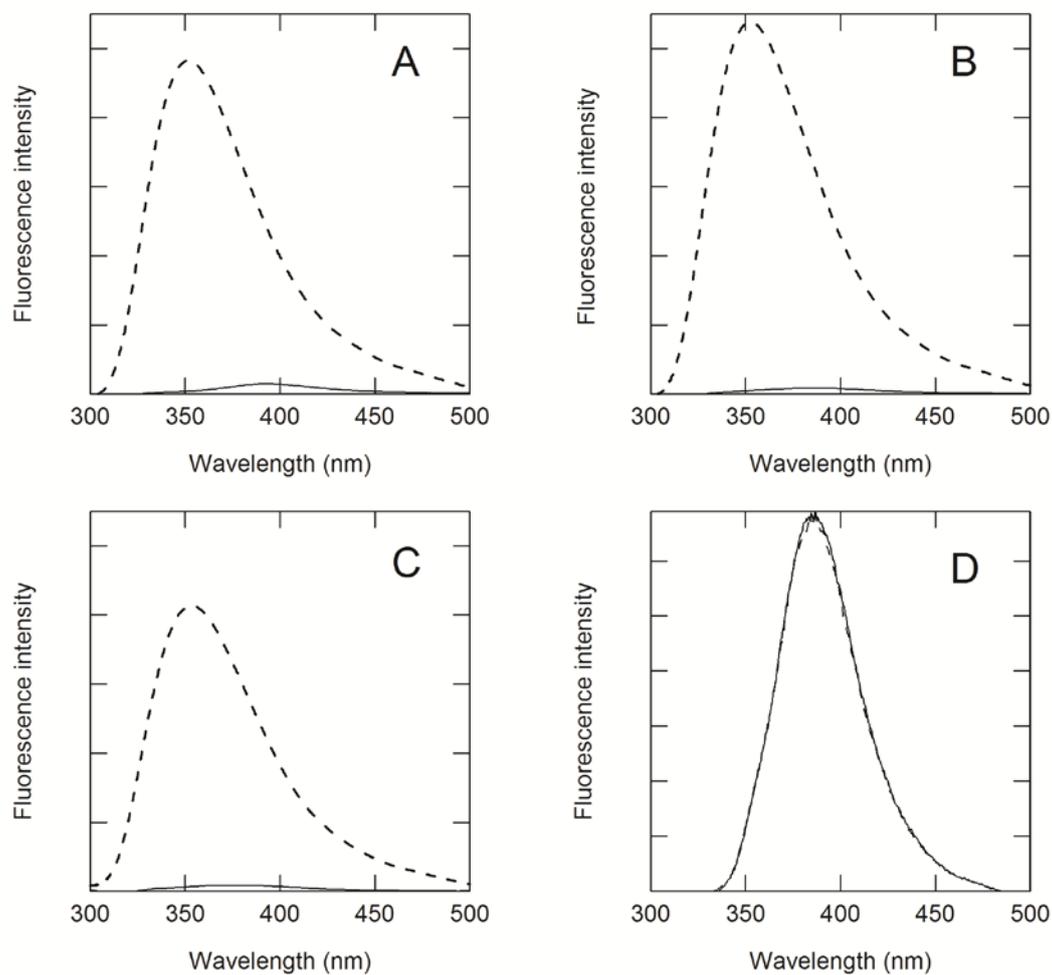


Figure S2. Fluorescence spectra of 3 μM octyl gallate (**C8**) (A), dodecyl gallate (**C12**) (B), cetyl gallate (**C16**) (C), and stearyl gallate (**C18**) (D) in the absence (solid lines) and presence (broken lines) of 10 μM DOPC. The excitation wavelength of the fluorescence spectra is 271 nm.