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Relationship between Antibacterial Activity of (+)-Catechin Derivatives and Their Interaction with a Model Membrane

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(+)-Catechin derivatives with different alkyl chain lengths were synthesized from (+)-catechin and various straight chain alkylaldehydes in the presence of methyl mercaptan, and their antibacterial activities against Gram-positive bacteria were evaluated. The antibacterial activity increased markedly with elongation of the alkyl chain lengths of the derivatives and reached a maximum at a chain of four to seven carbons. Subsequently, interaction of the (+)-catechin derivatives with a model membrane using liposome was investigated. The derivatives with a chain of three carbons or more were found to have very strong affinity for the membrane. The injury action of the derivatives against the membrane was examined with liposome in which calcein was enclosed as a fluorescent indicator. The leakage was observed in the derivatives with chain lengths of four carbons or more. Particularly the derivatives with chains longer than five carbons are considered to destroy the liposome membrane judging from the degree of the fluorescent leakage. These results implied that the lipophilicity and disrupting ability of the (+)-catechin derivatives to the liposome membrane participate in their antibacterial activity.

KEYWORDS: Antibacterial activity; (+)-catechin derivative; lipid bilayer; liposome

INTRODUCTION

Green tea contains various catechins such as (+)-catechin, (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECg), and (−)-epigallocatechin gallate (EGCg). Recently, they have been found to have various biological effects such as antimutagenicity (*1*, *2*), anticarcinogenicity (*3*), antitumorigenicity (*4*, *5*), antioxidant properties (*6*−*8*), and antihypercholesterolemia properties (*9*). The biological activities of catechins have been evaluated by in vitro experiments using cultured cells or bacteria, but the order of activity was variable. We developed a method of estimating the affinity of polyphenols for model membranes using liposome (*10*), and examined the interaction of tea catechins with the lipid bilayers. As a result, the order of the amount of tea catechins incorporated into the lipid bilayers was the same as that of the partition coefficients in a 1-octanol/phosphate-buffered saline (PBS) system. Furthermore, tea catechins with a galloyl moiety were located on the surface of the lipid bilayers, and perturbed the membrane structure. We found that the affinity of tea catechins for lipid bilayers was governed by the number of hydroxyl groups on the B-ring (e.g., EC > EGC, and ECg > EGCg), the presence of a galloyl moiety (e.g., ECg > EC, and EGCg > EGC), and the stereochemical structure of each catechin (e.g., ECg > (−)-catechin gallate, and EGCg > (−)-gallocatechin gallate) (*11*, *12*).

The antibacterial activity of various teas and tea catechins has been investigated as well (*13*−*20*). In these studies, it was found that tea catechins with a galloyl moiety have higher activity than those without a galloyl moiety. It has been suggested that the mechanism of antibacterial activity is associated with the membrane injury activity including effects on the membrane fluidity. Thus, the intensity of the antibacterial activity of tea catechins can partly be explained by our findings described above.

On the other hand, the antibacterial activity of tea catechins without a galloyl moiety (e.g., EC, EGC, (+)-catechin) is very weak (*13*). It has been reported that the antibacterial activity of EC derivatives with an alkyl chain is higher than that of EC (*14*, *21*). These studies using EC derivatives would indicate that the antibacterial activity was related to the partition coefficients of these derivatives.

We carried out the reaction of (+)-catechin with the various aldehydes in the presence of methyl mercaptan, and found that the derivatives with alkyl chains at the 6 and 8 positions of the A-ring of (+)-catechin can be simply synthesized. Further, we

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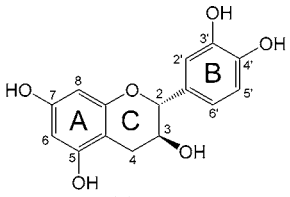

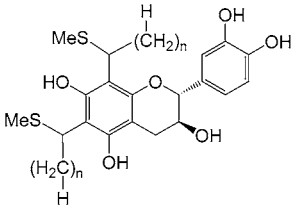

C 1 (n= 0) : (+)-6,8-bis-(1-methylthiomethyl)catechin
C 2 (n= 1) : (+)-6,8-bis-(1-methylthioethyl)catechin
C 3 (n= 2) : (+)-6,8-bis-(1-methylthiopropyl)catechin
C 4 (n= 3) : (+)-6,8-bis-(1-methylthiobutyl)catechin
C 5 (n= 4) : (+)-6,8-bis-(1-methylthiopentyl)catechin
C 6 (n= 5) : (+)-6,8-bis-(1-methylthiohexyl)catechin
C 7 (n= 6) : (+)-6,8-bis-(1-methylthioheptyl)catechin
C 8 (n= 7) : (+)-6,8-bis-(1-methylthiooctyl)catechin
C 9 (n= 8) : (+)-6,8-bis-(1-methylthiononyl)catechin
C10 (n= 9) : (+)-6,8-bis-(1-methylthiodecyl)catechin

Figure 1. Chemical structures of (+)-catechin and its derivatives.

evaluated the antibacterial activity of those (+)-catechin derivatives. Then, the interaction between those (+)-catechin derivatives and the lipid membranes was investigated using liposome as a model membrane to elucidate the mechanism of their antibacterial activity.

MATERIALS AND METHODS

Materials. (+)-Catechin was purchased from Aldrich (Milwaukee, WI). Phosphatidylcholine from egg yolk (egg PC) was obtained from Nippon Fine Chemicals (Hyogo, Japan). Formaldehyde, propionaldehyde, and hexanal were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Acetaldehyde and octanal were obtained from Merck (Darmstadt, Germany). Butylaldehyde, pentanal, heptanal, nonanal, and decanal were purchased from Kanto Chemical Co. (Tokyo, Japan). Methyl mercaptan sodium salt was obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Calcein was purchased from Molecular Probes (Eugene, OR).

Syntheses of (+)-Catechin Derivatives. We synthesized 10 kinds of (+)-catechin derivatives from (+)-catechin and aldehydes with various alkyl chain lengths, formaldehyde, acetaldehyde, propionaldehyde, butyraldehyde, pentanal, hexanal, heptanal, octanal, nonanal, and decanal. These (+)-catechin derivatives were termed **C1**−**C10** according to the carbon number of the reacted aldehyde (**Figure 1**). **Figure 2** shows the synthetic method of **C3**, (+)-6,8-bis-1-methylthiopropyl-catechin, as an example.

The synthetic reactions and purifications were carried out separately for each aldehyde. (+)-Catechin (290 mg, 1 mmol) was added to a reaction mixture of aldehyde (20 mmol), 15% methyl mercaptan sodium salt aqueous solution (10 mL, 21.4 mmol), acetonitrile (160 mL), H_2O (30 mL), and HCl (6.65 mL) with stirring. The mixture was kept for 6 h at room temperature with vigorous stirring and extracted with EtOAc (500 mL). The organic layer was washed twice with H_2O (100 mL), dehydrated with $MgSO_4$ overnight, and concentrated in vacuo. The obtained pale yellow oily material was purified by preparative HPLC with a Jasco liquid chromatograph apparatus (Tokyo, Japan). The conditions were as follows: column, CAPCELL PAK C18 UG120, 20 × 250 mm (Shiseido Co., Ltd., Japan); mobile phase, H_2O−MeCN (the proportions of MeCN were 27.5% for **C1**, 55% for **C2**, 58% for **C3**, 63% for **C4**, 70% for **C5**, 77% for **C6**, 84% for **C7**, 90% for **C8**, 95% for **C9**, and 98% for **C10**, respectively); temperature, room temperature; detection, 280 nm; flow rate, 10 mL/min. The eluate was concentrated in vacuo to yield reactive products **C1** (96.5 mg, 23.5%), **C2** (304.7 mg, 69.5%), **C3** (245.1 mg, 52.5%), **C4** (353.5 mg, 71.5%), **C5** (384.3 mg, 73.5%), **C6** (80.6 mg, 80.6%), **C7** (337.5 mg, 58.3%), **C8** (466.6 mg, 76.9%), **C9** (341.0 mg, 53.7%), and **C10** (471.6 mg, 71.1%). The analytical HPLC conditions were as follows: column, Mightysil RP8, 4.6 × 150 mm (Kanto Chemical Co., Japan); mobile phase, 0.05% H_3PO_4 in H_2O−MeCN (the proportions of MeCN were 30% for **C1**, 50% for **C2**, 60% for **C3** and **C4**, 70% for **C5** and **C6**, 80% for **C7** and **C8**, and 90% for **C9** and **C10**, respectively); temperature, 40 °C; detection, 280 nm; flow rate, 1 mL/min.

Antibacterial Activity. The antibacterial activity of (+)-catechin derivatives was assayed by the liquid dilution method (*22*) against six kinds of Gram-positive bacteria: *Bacillus cereus* (JCM2152), *Bacillus circulans* (IFO13626), *Bacillus subtilis* (IAM12118), *Staphylococcus aureus* (IAM1011), *Staphylococcus xylosus* (ATCC29971), and *Enterococcus faecalis* (JCM5803). Bacterial suspensions for inocula were prepared from overnight culture on brain heart infusion agar (Nissui Pharmaceutical Co., Ltd., Japan) suspended in physiological saline to give a concentration at 10^6 to 10^7 cfu/mL. Test compound solutions (10 μL), which were diluted with 50% DMSO, were added to the 96-well microtiter plate with 85 μL of Mueller−Hinton broth (Merck,

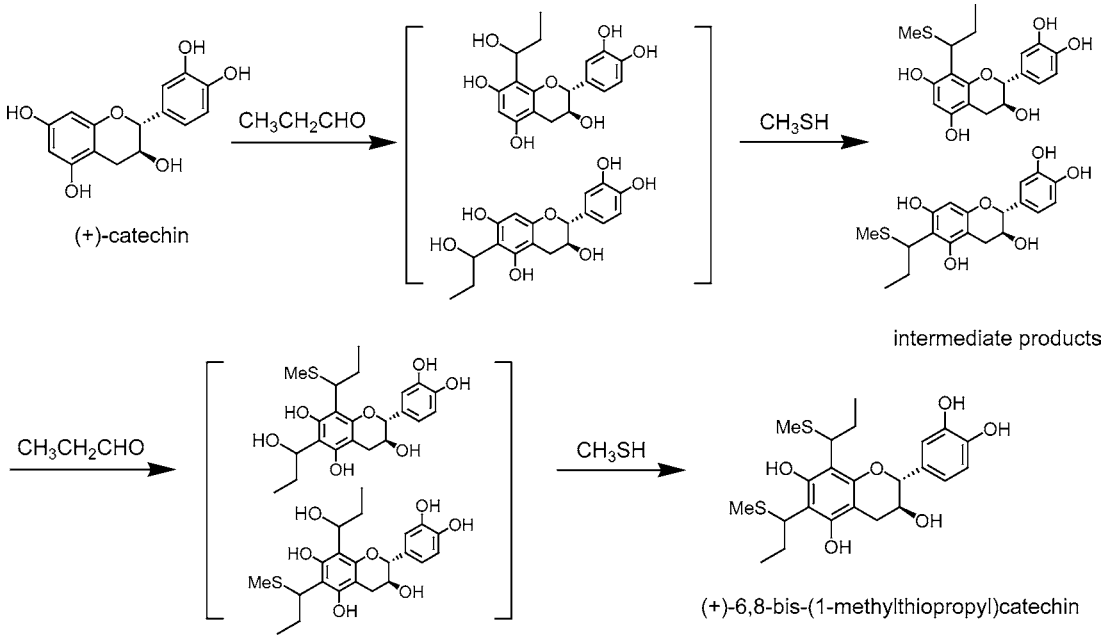

Figure 2. Proposed synthetic reaction for (+)-6,8-bis(1-methylthiopropyl)catechin (**C3**).

Germany). Then 5 μL of the bacterial suspensions were inoculated into the wells and incubated for 24 h at 35 °C. The final concentration of DMSO in the medium was 5%, and controls were also prepared containing DMSO at this concentration. We confirmed that the presence of 5% DMSO has no influence on growth of bacteria. The minimum inhibitory concentration (MIC) was determined as the lowest concentration preventing visible growth.

Incorporation into Liposome. The affinities of the synthesized compounds for lipid bilayers was measured as previously reported (*12*). Briefly, the thin film of egg PC on the inner surface of the flask was dried with a vacuum pump. An aqueous glucose solution (300 mM) was then poured into the flask, and the mixture was sonicated in an ultrasonicator. The resulting solution of multilamellar vesicles was then sonicated in a cup-horn type of sonicator to change the multilamellar vesicles to small unilamellar vesicles. The liposomal solution was diluted 10-fold with PBS. The final concentration of egg PC in the liposomal solution was adjusted to 1 mg/mL. Each (+)-catechin derivative solution in ethanol was added to the liposomal solution. The final concentration of (+)-catechin derivative was 50 μM containing 10% ethanol. We confirmed that the presence of 10% ethanol has no influence on the liposomes (data not shown). The amount of the compound in the solution was measured by HPLC, and the proportion (%) of the compound incorporated into the lipid bilayers was calculated as follows:

$$[\text{amount incorporated}]/[\text{amount added}] \times 100$$

Partition Coefficients. The partition coefficients of synthesized compounds were measured as previously reported (*12*). Each derivative solution in 1-octanol (100 μM, 2 mL) was vigorously mixed with 2 mL of water. After centrifugation at 200 *g* for 10 min, the amount of the derivative in each layer was measured by HPLC. The results were expressed as common logarithms.

Analysis of Calcein Leaking from Calcein-Trapped Liposomes. The membrane injury action of (+)-catechin derivatives was examined with model membranes using calcein-trapped liposomes. The enclosed fluorescence substance, calcein, leaks out by the destruction of membrane structure. The leakage of calcein was measured as previously reported (*12*). Briefly, the liposome with a dense internal aqueous phase containing calcein was prepared. The designated amount of a (+)-catechin derivative was added to the liposomal solution, and the resulting solution was incubated for 1 h at 20 °C. Fluorescence of calcein leaking from the internal aqueous phase to the external medium was measured with excitation at 445 nm and emission at 510 nm. The degree of calcein leakage was calculated as the percent of the fluorescence intensity of completely released calcein from the liposomes after treatment with 1% Triton X-100.

Identification of (+)-Catechin Derivatives. High-resolution fast atom bombardment mass spectrometry (HR-FABMS) spectra were taken on a JEOL JMS-700 mass spectrometer. ^{1}H NMR spectra were recorded on a JEOL GSX-500 or α-400 spectrometer using tetramethylsilane (TMS) as an internal standard.

Data for (+)-6,8-bis(1-methylthiomethyl)catechin (C1): colorless powder; HR-FABMS *m/z* 433.0777 $[M + Na]^+$ (calcd for $C_{19}H_{22}O_6S_2$-Na, 433.0757); ^{1}H NMR (acetone-d_6, 500 MHz) δ 1.98 (3H, s, SCH_3a), 1.99 (3H, s, SCH_3b), 2.62 (1H, dd, J = 8.9, 16.0 Hz, H-4a), 2.99 (1H, dd, J = 5.5, 16.0 Hz, H-4b), 3.72 (2H, d, J = 8.1 Hz, CH_2a), 3.78 (2H, d, J = 8.1 Hz, CH_2b), 3.86 (2H, d, J = 8.1 Hz, CH_2a′), 3.89 (2H, d, J = 8.1 Hz, CH_2b′), 3.99 (1H, ddd, J = 5.5, 7.9, 8.9 Hz, H-3), 4.57 (1H, d, J = 7.9 Hz, H-2), 6.79 (1H, dd, J = 1.9, 8.1 Hz, H-6′), 6.81 (1H, d, J = 8.1 Hz, H-5′), 6.94 (1H, d, J = 1.9 Hz, H-2′).

Data for (+)-6,8-bis(1-methylthioethyl)catechin (C2): light yellow powder; HR-FABMS *m/z* 461.1017 $[M + Na]^+$ (calcd for $C_{21}H_{26}O_6S_2$-Na, 461.1070); ^{1}H NMR (acetone-d_6, 500 MHz) δ 1.44−1.53 (6H, d, J = 7.0 Hz, CH_3), 1.90−1.99 (6H, s, SCH_3), 2.54−2.62 (1H, dd, J = 8.6, 16.1 Hz, H-4a), 2.94−3.04 (1H, dd, J = 6.1, 16.1 Hz, H-4b), 3.93−4.00 (1H, m, H-3), 4.52−4.61 (1H, d, J = 8.0 Hz, H-2), 4.63−4.81 (2H, q, J = 7.2 Hz, CH), 6.75−6.78 (1H, dd, J = 3.0, 8.1 Hz, H-6′), 6.79−6.83 (1H, d, J = 8.2 Hz, H-5′), 6.91−6.94 (1H, d, J = 2.1 Hz, H-2′).

Data for (+)-6,8-bis(1−methylthiopropyl)catechin (C3): yellow powder; HR-FABMS *m/z* 489.1311 $[M + Na]^+$ (calcd for $C_{23}H_{30}O_6S_2$-Na, 489.1383); ^{1}H NMR (acetone-d_6, 400 MHz) δ 0.81−0.97 (6H, CH_3), 1.75−1.86 (4H, CH_2), 1.88−1.97 (6H, SCH_3), 2.53−2.64 (1H, H-4a), 2.96−3.10 (1H, H-4b), 3.88−3.97 (1H, H-3), 4.48−4.54 (1H, H-2), 4.56−4.62 (2H, CH), 6.76−6.80 (1H, H-6′), 6.81−6.83 (1H, H-5′), 6.91−6.94 (1H, H-2′).

Data for (+)-6,8-bis(1-methylthiobutyl)catechin (C4): light brown powder; HR-FABMS *m/z* 517.1773 $[M + Na]^+$ (calcd for $C_{25}H_{34}O_6S_2$-Na, 517.1696); ^{1}H NMR (acetone-d_6, 400 MHz) δ 0.78−0.93 (6H, CH_3), 1.21−1.45 (4H, 3″,3‴-CH_2), 1.65−1.83 (4H, 2″,2‴-CH_2), 1.87−1.96 (6H, SCH_3), 2.49−2.63 (1H, H-4a), 2.97−3.10 (1H, H-4b), 3.89−3.98 (1H, H-3), 4.47−4.56 (1H, H-2), 4.58−4.72 (2H, CH), 6.76−6.80 (1H, H-6′), 6.80−6.83 (1H, H-5′), 6.92−6.94 (1H, H-2′).

Data for (+)-6,8-bis(1-methylthiopentyl)catechin (C5): light brown powder; HR-FABMS *m/z* 545.2047 $[M + Na]^+$ (calcd for $C_{27}H_{38}O_6S_2$-Na, 545.2009); ^{1}H NMR (acetone-d_6, 400 MHz) δ 0.77−0.98 (6H, CH_3), 1.17−1.51 (4H, 3″,4″,3‴,4‴-CH_2), 1.70−1.84 (4H, 2″,2‴-CH_2), 1.87−1.97 (6H, SCH_3), 2.53−2.64 (1H, H-4a), 2.97−3.10 (1H, H-4b), 3.90−4.03 (1H, H-3), 4.48−4.56 (1H, H-2), 4.58−4.71 (2H, CH), 6.75−6.80 (1H, H-6′), 6.81−6.83 (1H, H-5′), 6.90−6.93 (1H, H-2′).

Data for (+)-6,8-bis(1-methylthiohexyl)catechin (C6): brown crystal; HR-FABMS *m/z* 551.2485 $[M + H]^+$ (calcd for $C_{29}H_{43}O_6S_2$, 551.2503); ^{1}H NMR (acetone-d_6, 400 MHz) δ 0.75−0.97 (6H, CH_3), 1.19−1.57 (12H, 3″,4″,5″,3‴,4‴,5‴-CH_2), 1.68−1.83 (4H, 2″,2‴-CH_2), 1.87−1.97 (6H, SCH_3), 2.53−2.64 (1H, H-4a), 2.97−3.10 (1H, H-4b), 3.80−4.04 (1H, H-3), 4.48−4.55 (1H, H-2), 4.57−4.75 (2H, CH), 6.72−6.80 (1H, H-6′), 6.81−6.82 (1H, H-5′), 6.90−6.94 (1H, H-2′).

Data for (+)-6,8-bis(1-methylthioheptyl)catechin (C7): brown resin; HR-FABMS *m/z* 601.2576 $[M + Na]^+$ (calcd for $C_{31}H_{46}O_6S_2$-Na, 601.2636); ^{1}H NMR (acetone-d_6, 400 MHz) δ 0.82−0.89 (6H, CH_3), 1.26−1.61 (16H, 3″,4″,5″,6″,3‴,4‴,5‴,6‴-CH_2), 1.71−1.74 (4H, 2″,2‴-CH_2), 1.87−1.97 (6H, SCH_3), 2.52−2.64 (1H, H-4a), 2.96–3.13 (1H, H-4b), 3.91–3.99 (1H, H-3), 4.48–4.55 (1H, H-2), 4.57−4.71 (2H, CH), 6.77−6.80 (1H, H-6′), 6.81−6.82 (1H, H-5′), 6.89−6.93 (1H, H-2′).

Data for (+)-6,8-bis(1-methylthiooctyl)catechin (C8): brown resin; HR-FABMS *m/z* 607.3168 $[M + H]^+$ (calcd for $C_{33}H_{51}O_6S_2$, 607.3129); ^{1}H NMR (acetone-d_6, 400 MHz) δ 0.85−0.89 (2H, CH_3), 1.19−1.62 (20H, 3″,4″,5″,6″,7″,3‴,4‴,5‴,6‴,7‴-CH_2), 1.69−1.76 (4H, 2″,2‴-CH_2), 1.87−1.97 (6H, SCH_3), 2.52−2.64 (1H, H-4a), 2.99−3.14 (1H, H-4b), 3.91−4.13 (1H, H-3), 4.53−4.62 (1H, H-2), 4.65−4.74 (2H, CH), 6.77−6.79 (1H, H-6′), 6.81−6.83 (1H, H-5′), 6.89−6.97 (1H, H-2′).

Data for (+)-6,8-bis(1-methylthiononyl)catechin (C9): brown resin; HR-FABMS *m/z* 657.3218 $[M + Na]^+$ (calcd for $C_{35}H_{54}O_6S_2$-Na, 657.3262); ^{1}H NMR (acetone-d_6, 400 MHz) δ 0.84−0.88 (6H, CH_3), 1.20−1.29 (24H, 3″,4″,5″,6″,7″,8″,3‴,4‴,5‴,6‴,7‴,8‴-CH_2), 1.71−1.77 (4H, 2″,2‴-CH_2), 1.87−1.97 (6H, SCH_3), 2.52−2.64 (1H, H-4a), 2.98−3.06 (1H, H-4b), 3.87−4.03 (1H, H-3), 4.40−4.55 (1H, H-2), 4.56−4.74 (2H, CH), 6.74−6.79 (1H, H-6′), 6.79−6.82 (4H, H-5′), 6.88−6.93 (4H, H-2′).

Data for (+)-6,8-bis(1-methylthiodecyl)catechin (C10): dark brown resin; HR-FABMS *m/z* 685.3527 $[M + Na]^+$ (calcd for $C_{37}H_{58}O_6S_2$-Na, 685.3575); ^{1}H NMR (acetone-d_6, 400 MHz) δ 0.87−0.88 (6H, CH_3), 1.27−1.29 (28H, 3″,4″,5″,6″,7″,8″,9″,3‴,4‴,5‴,6‴,7‴,8‴,9‴-CH_2), 1.83−1.88 (4H, 2″,2‴-CH_2), 1.89−1.98 (6H, SCH_3), 2.53−2.64 (1H, H-4a), 2.89−3.14 (1H, H-4b), 3.84−3.99 (1H, H-3), 4.47−4.51 (1H, H-2), 4.53−4.74 (2H, CH), 6.76−6.79 (1H, H-6′), 6.79−6.81 (1H, H-5′), 6.87−6.94 (1H, H-2′).

^{1}H NMR assignments of compounds **C2**−**C10** were not established completely because of the presence of four stereoisomers in the test samples.

RESULTS

Ten kinds of (+)-catechin derivatives were synthesized using the various aldehydes (**Figure 1**). These (+)-catechin derivatives other than **C1** have two asymmetric carbons attached to *S*-methyl groups in the alkyl chains at the 6 and 8 positions of the A

Table 1. Minimum Inhibitory Concentration of (+)-Catechin Derivatives against Gram-Positive Bacteria (μg/mL)

test compd	MIC[a] (μg/mL)					
	B. cereus, JCM 2152	*B. circulans*, IFO 13626	*B. subtilis*, IAM 12118	*S. aureus*, IAM 1011	*S. xylosus*, ATCC 29971	*E. faecalis*, JCM 5803
(+)-catechin	6400	12800	12800	12800	6400	6400
C1	>400	>400	>400	>400	>400	>400
C2	200	400	200	400	>400	>400
C3	50	50	50	100	200	400
C4	25	25	25	25	50	50
C5	12.5	12.5	12.5	25	25	25
C6	12.5	12.5	12.5	25	12.5	12.5
C7	25	25	25	100	12.5	12.5
C8	100	100	100	>100	50	50
C9	>100	>100	>100	>100	>100	>100
C10	>100	>100	>100	>100	>100	>100

[a] MICs were determined by the liquid medium dilution method in Mueller–Hinton broth supplement.

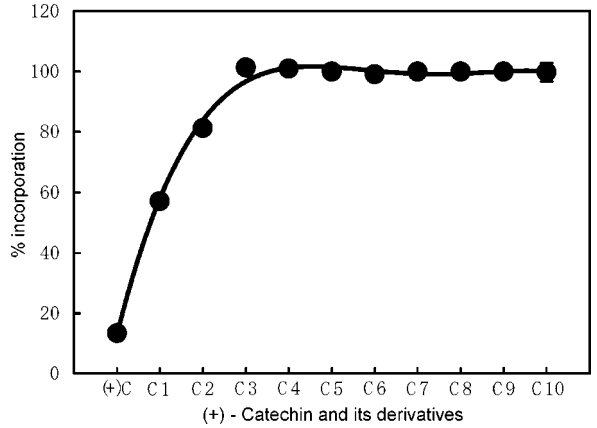

Figure 3. Percent incorporation of (+)-catechin derivatives into liposome. A (+)-catechin derivative added to liposomal solution was incubated for 20 min at 20 °C and centrifuged. The percent incorporation of (+)-catechin derivatives into the liposomes was measured by HPLC. Each result is shown as the mean value of four independent experiments with the SD.

ring, and thus four stereoisomers exist. Since these stereoisomers could not be separated completely, we used the mixture of these stereoisomers in this study.

We evaluated the antibacterial activity of the synthesized (+)-catechin derivatives against Gram-positive bacteria (**Table 1**). The MIC of (+)-catechin for all tested bacteria was above 6400 μg/mL. The MIC decreased with elongation of the alkyl chain lengths of the derivatives **C1**−**C4**. The antibacterial activities of compounds **C4**−**C7** were very strong. In the case of compounds **C8**−**C10**, we could not evaluate the exact antibacterial activity at higher concentration than 100 μg/mL, because of their low solubility in the medium. Accordingly MICs for these compounds were expressed as >100 μg/mL. The (+)-catechin derivatives prepared in this study had no activity against Gram-negative bacteria (data not shown).

Figure 3 shows the percent incorporation of (+)-catechin derivatives into liposome with lipid bilayers as the model membrane. The amount of (+)-catechin incorporated into the lipid bilayers was about 13%. In contrast, the amount of (+)-catechin derivatives incorporated into the liposome increased with elongation of the alkyl chain lengths, and the percent incorporation of the derivatives with straight chains of three carbons or more was almost 100%. These results indicated that the introduction of alkyl chains to (+)-catechin increased the affinity for the liposome membrane. We also examined the partition coefficient of each (+)-catechin derivative in the 1-octanol/water system (**Table 2**). The partition coefficients of the derivatives increased with elongation of the alkyl chain lengths. Thus, the order of the partition coefficient was closely correlated with the amount of derivatives incorporated into the lipid bilayers, which is attributable to their lipophilicity.

Table 2. Partition Coefficients of (+)-Catechin Derivatives Evaluated with the 1-Octanol/Water System[a]

test compd	log *P*	test compd	log *P*	test compd	log *P*
(+)-catechin	0.38 ± 0.00	C4	3.56 ± 0.00	C8	–
C1	2.19 ± 0.00	C5	3.65 ± 0.00	C9	–
C2	3.32 ± 0.01	C6	–	C10	–
C3	3.45 ± 0.00	C7	–		

[a] The partition coefficient was calculated by dividing the amount of each sample in 1-octanol by that in water after vigorously mixing and centrifuging a mixture containing 50 μM sample. The results are shown as the mean value of four independent experiments with the SD.

Figure 4 shows the effects of (+)-catechin derivatives on calcein leakage from liposomes. In our preliminary experiments, we confirmed that the fluorescence emission spectrum of each derivative (100 μM) and that of calcein (100 μM) did not overlap (data not shown). (+)-Catechin and its derivatives, **C1**−**C3**, did not cause any calcein leakage from liposomes even at high concentrations (**Figure 4A**). Calcein did not leak at low concentrations of **C4**, but leaked in a dose-dependent manner at higher concentrations (**Figure 4A**). The derivatives with longer carbon chains, **C5**−**C10**, dose-dependently promoted the leakage of the calcein (**Figure 4B**). The effects of 10 μM (+)-catechin derivatives with longer carbon chains, **C5**−**C10**, on the calcein leakage are shown as a bar graph for clear comparison (**Figure 4C**). Among the six compounds, **C5** showed the lowest activity for calcein leakage, followed by **C6**. At concentrations of 60 and 80 μM, the ratio of calcein leakage increased in the following order: **C1**−**C3** < **C4** < **C5** < **C6**−**C10** (**Figure 4A,B**).

Several parameters investigated in the present study can be summarized as follows: (antibacterial activity) (+)-catechin < **C1** < **C2** < **C3** < **C4** < **C5**−**C7**; (amount incorporated into the lipid bilayers) (+)-catechin < **C1** < **C2** < **C3**−**C7**; (partition coefficients) (+)-catechin < **C1** < **C2** < **C3** < **C4** < **C5** < **C6**, **C7**; (ratio of calcein leakage) (+)-catechin, **C1**−**C3** < **C4** < **C5** < **C6**, **C7**.

DISCUSSION

In this study, we synthesized (+)-catechin derivatives and evaluated their antibacterial activity against Gram-positive

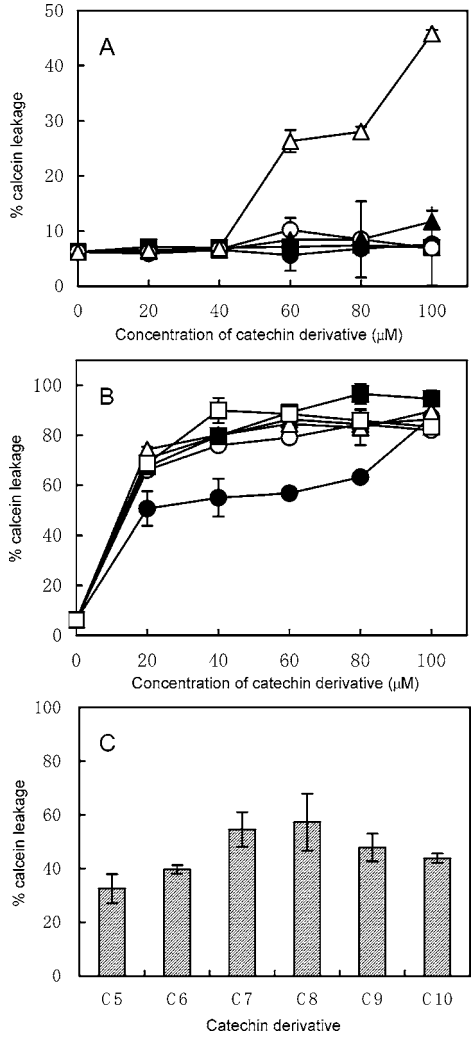

Figure 4. Effects of (+)-catechin derivatives on calcein leakage. A (+)-catechin derivative added to liposomal solution was incubated for 1 h at 20 °C and centrifuged. The fluorescence intensity of calcein in the external medium was measured. Each result is shown as the mean value of four independent experiments with the SD. (A) Catechin derivatives with a shorter carbon chain: (■) (+)-catechin, (●) **C1**, (○) **C2**, (▲) **C3**, and (△) **C4**. (B) Catechin derivatives with a longer carbon chain: (●) **C5**, (○) **C6**, (▲) **C7**, (△) **C8**, (■) **C9**, and (□) **C10**. (C) Percent calcein leakage at a low concentration (10 μM) of catechin derivatives with a longer carbon chain.

bacteria. Further, we investigated the interaction of the derivatives with liposome as a model membrane.

It has been reported that (+)-catechin reacts with aldehyde at the 6 and 8 positions of the A-ring to form polymers by cross-linking reactions (*23*−*25*, *27*, *28*). These polymers are decomposed by thiols to give a catechin monomer substituted by an alkyl sulfide group at the cross-linkage position (*24*). Furthermore, the reaction of EGCg with formaldehyde in the presence of alkyl thiol was reported to synthesize EGCg derivatives with various lengths of the alkyl chain, which is derived from the thiol compound (*26*).

In the present study, we synthesized (+)-catechin derivatives, named **C1**−**C10**, with various lengths of alkyl chains at the 6 and 8 positions, which are derived from aldehydes by the reaction of (+)-catechin and aldehydes in the presence of methyl mercaptan sodium salt as shown in **Figure 1**.

The percent incorporation of the (+)-catechin derivatives into liposomes increased with elongation of the alkyl chain lengths of the derivatives (**Figure 3**). The antibacterial activity of (+)-catechin derivatives also increased with elongation of the alkyl chain lengths of the derivatives (**Table 1**). Thus, we presume that the antibacterial activity of the (+)-catechin derivatives and their interaction with the bacterial membrane are closely correlated. The percent incorporation of **C3**−**C10** into the liposomes was almost 100%, but the antibacterial activity of **C3** was weaker than those of **C4**−**C7**. **Figure 4** shows that **C4** and higher caused calcein leakage, but **C3** did not cause calcein leakage. Thus, it is presumed that the antibacterial activity of the (+)-catechin derivatives is associated with both the affinity for the lipid bilayer and the disruption of the membrane structure. The mechanism of antibacterial activity is hypothesized to be as follows: These compounds adsorb onto the membrane surface of the bacteria and deteriorate the membrane function. Consequently, the observed antibacterial activity of the (+)-catechin derivatives in this study is reinforced by the addition of the alkyl chains, because the presence of the alkyl chains accelerated the adsorption on the surface of the membrane and the disruption of the membrane by increasing the lipophilicity.

The (+)-catechin derivatives prepared in this study had no activity against Gram-negative bacteria (data not shown). This would be attributable to the membrane structures of Gram-negative bacteria. It has already been reported that the low susceptibility of Gram-negative bacteria for tea catechins may be due partially to the presence of a strong negative charge of lipopolysaccharide at the exterior of the outer membrane (*29*, *30*). This charge repels the negative phenolate ion formed from the catechins at physiological pH.

Here the antibacterial activity is termed a biological parameter, and the amount incorporated into the lipid bilayers, the partition coefficients, and the ratio of calcein leakage are termed chemical parameters. We assumed the biological parameter should be correlated with the three chemical parameters. Among the chemical parameters, the amount incorporated into the lipid bilayers and the ratio of calcein leakage should be important. Neither the amount incorporated into the lipid bilayers nor the ratio of calcein leakage can singly explain the difference in antibacterial activities. We suppose that, in addition to the amount incorporated into the lipid bilayers, the activity disrupting membrane structure should affect the antibacterial activity of (+)-catechin derivatives such as **C3**, **C4**, **C5**, **C6**, and **C7**.

ABBREVIATIONS USED

C1, (+)-6,8-bis(1-methylthiomethyl)catechin; **C2**, (+)-6,8-bis(1-methylthioethyl)catechin; **C3**, (+)-6,8-bis(1-methylthiopropyl)catechin; **C4**, (+)-6,8-bis(1-methylthiobutyl)catechin; **C5**, (+)-6,8-bis(1-methylthiopentyl)catechin; **C6**, (+)-6,8-bis(1-methylthiohexyl)catechin; **C7**, (+)-6,8-bis(1-methylthioheptyl)catechin; **C8**, (+)-6,8-bis(1-methylthiooctyl)catechin; **C9**, (+)-6,8-bis(1-methylthiononyl)catechin; **C10**, (+)-6,8-bis(1-methylthiodecyl)catechin; egg PC, phosphatidylcholine from egg yolk; HR-FABMS, high-resolution fast atom bombardment mass spectrometry; MIC, minimum inhibitory concentration; PBS, phosphate-buffered saline.

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