Evaluation of Antibacterial Activity Expression of the Hinokitiol/Cyclodextrin Complex Against Bacteria

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ABSTRACT: The purpose of this study was to assess the antimicrobial activity of a solid dispersion prepared by mixing and grinding hinokitiol (HT) with α-cyclodextrin (αCD), β-cyclodextrin (βCD), or γ-cyclodextrin (γCD). Antimicrobial activity was evaluated by calculating the minimum inhibitory concentration (MIC) and evaluating the change in the number of bacteria over time. The test microbes used were two Gram-positive bacteria (Bacillus subtilis and Staphylococcus aureus), two Gram-negative bacteria (Escherichia coli and Pseudomonas aeruginosa), and two fungi (Candida albicans and Aspergillus brasiliensis). Calculation of the MIC value of HT using the agar dilution method revealed that the MIC of HT/CD inclusion complexes was lower than that of HT alone. HT irreversibly inhibited the growth of microorganisms in a short amount of time. HT/CD complexes retained the antimicrobial activity of HT as a result of including HT in a CD complex. These results suggest that inclusion of HT, an antimicrobial component, using CDs could lead to appropriate control of the drug release rate and efficient display of antimicrobial activity.

1. INTRODUCTION

Natural flavors are extracted from volatile compounds obtained from plants.1 These compounds are generally used as flavoring agents or fragrances in foods and cosmetics, and they act as antibacterials, preservatives, or insecticides.2 Drug-resistant bacteria have become more prevalent around the world, and attention has focused again on fragrances that have long been studied for their antibacterial activity. Many fragrances are natural and also have a broad antibacterial spectrum. Fragrances are reported to have antibacterial activity by themselves as well as a high level of antibacterial activity when used in combination with antibiotics.3 Thus, fragrances will presumably be used as a new antimicrobial ingredient in pharmaceuticals. However, fragrances are poorly soluble in water, volatile, unstable, and highly reactive, so they often need to be specially formulated.

Hinokitiol (HT) is a phenolic monoterpene (Figure 1A). It is reported to have antibacterial, antifungal, antiviral, and anti-inflammatory actions.4-8 HT is used in a wide range of fields, such as pharmaceuticals, foods, and household goods; if it readily exhibits a wide variety of activities, its use will expand further in the future.9 However, HT has the sublimation characteristics of a fragrance, and the fact that it is poorly soluble in water is a concern. In addition, HT is susceptible to external conditions such as photodecomposition, polymerization, and condensation, limiting the utility of HT in terms of its antimicrobial activity. Since these properties hinder the development of products using HT, the water solubility of HT and heat and light stability of HT need to be improved. In recent years, studies on the formulation of antibacterial substances using various additives have been reported. Cyclodextrin encapsulation technology is a means to prevent the development of resistant bacteria in that it enables the
antimicrobial activity of guest molecules in a smaller amount and suppresses the use of unnecessary antimicrobial substances. The current authors previously reported complex formation using HT and cyclodextrin (CD).\textsuperscript{10,11} Formation of a complex by HT and α, β, or γCD results in different inclusion behaviors as well as different dissolution behaviors of HT in water.

Cyclodextrin (CD) is a compound in which D-glucopyranose is cyclically linked by α-1,4 bonds; CD is classified as α cyclodextrin (αCD), β-cyclodextrin (βCD), or γ-cyclodextrin (γCD) depending on whether it contains 6, 7, or 8 glucopyranoses. CDs have been used as host molecules to form inclusion compounds (Figure 1B–D). CD is known to have a hydrophilic rim at the entrance of the ring and outside but a hydrophobic inner cavity; this allows it to incorporate various hydrophobic guest molecules into its cavity to form inclusion complexes.\textsuperscript{12} CDs are used in various fields since the inclusion of guest molecules should improve solubility,\textsuperscript{13} stability,\textsuperscript{14} antibacterial action,\textsuperscript{15} and antioxidant action\textsuperscript{16} as a result of the effects of physicochemical properties. The formation of a complex between an antibiotic and CD is expected to improve solubility, improve bioavailability by improving drug permeability to the bacterial membrane barrier, and change antibacterial activity and chemical stability. CD enables efficient and stable supply of antibacterial activity and is a useful material for the development of pharmaceuticals and daily necessities. Several studies have improved the antibacterial activity of fragrances by complexing them with CDs.\textsuperscript{17,18} As an example, inclusion of guava leaf oil in hydroxypropyl-β-cyclodextrin (HP/βCD) improved the antibacterial activity of HP/βCD by improving its stability.\textsuperscript{19} The current authors previously reported the effect of formation of HT/γCD inclusion complexes on antibacterial activity.\textsuperscript{11} However, the detailed mechanism of the effect of inclusion in CD on the antimicrobial activity of HT has not been investigated. One of the purposes of this study is the evaluation of \textit{Escherichia coli}, a Gram-negative bacterium. \textit{E. coli} is present in the moist environment of community hospitals, as well as in the human skin and intestinal tract. Therefore, exogenous infections derived from the environment and endogenous infections caused by indigenous bacteria of patients themselves are regarded as factors causing hospital infections. It is interesting that the HT/CD complex laid the foundation for an antibacterial mechanism against \textit{E. coli}. We hope to make progress in research on Gram-positive bacterial species of HT/CD complexes. To expand the use of HT, the current study examined whether HT/CD inclusion complexes were formed as a result of cogrinding. Moreover, this study examined the effects of the formation of HT/CD inclusion complexes on the antimicrobial activity of HT and its mechanism of action.

2. RESULTS AND DISCUSSION

2.1. Antibacterial Testing. An antimicrobial test using the agar dilution method was performed with four microbes, and MICs were calculated (Table 1) to assess the antimicrobial activity of HT.

The MIC of HT alone was 80 μg/mL with respect to \textit{B. subtilis}, 160 μg/mL with respect to \textit{S. aureus}, 80 μg/mL with respect to \textit{E. coli}, and 320 μg/mL with respect to \textit{P. aeruginosa}. Ground HT alone had an MIC similar to that of HT crystals.\textsuperscript{11} The PM of HT and αCD (molar ratio = 1:2), the PM of HT and βCD (molar ratio = 1:1), and the PM of HT and γCD (molar ratio = 1:1) had decreased MICs with respect to dia. However, the GM of HT and αCD (molar ratio = 1:2), the GM of HT and βCD (molar ratio = 1:1), and the GM of HT and γCD (molar ratio = 1:1) had MICs of 20 μg/mL with respect to \textit{B. subtilis}, 40 μg/mL with respect to \textit{S. aureus}, and 20 μg/mL with respect to \textit{E. coli}; antimicrobial activity increased about fourfold compared to that of HT alone.

| samples          | MIC (μg/mL) |
|------------------|-------------|
| B. sub | S. a | E. coli | P. aer |
| CDs    | n.d. | n.d. | n.d. | n.d. |
| HT intact | 80 | 160 | 80 | 320 |
| HT ground | 40 | 40 | 80 | 320 |
| PM (HT/αCD = 1:2) | 40 | 80 | 80 | 320 |
| PM (HT/βCD = 1:1) | 40 | 80 | 80 | 320 |
| PM (HT/γCD = 1:1)* | 40 | 80 | 80 | 320 |
| GM (HT/αCD = 1:2) | 20 | 40 | 20 | 160 |
| GM (HT/βCD = 1:1) | 20 | 40 | 20 | 160 |
| GM (HT/γCD = 1:1)** | 20 | 40 | 20 | 160 |

*\textit{B. sub: Bacillus subtilis}, \textit{E. coli: Escherichia coli}, \textit{S. a: Staphylococcus aureus}, \textit{P. aer: Pseudomonas aeruginosa}. **All MIC values are converted into HT concentration. n.d.: not determined. *Values from Suzuki et al., 2015.
of HT and γCD (molar ratio = 1:1) had MICs of 20 μg/mL with respect to C. albicans and 20 μg/mL with respect to A. brasiliensis. In contrast, the GM of HT and αCD (molar ratio = 1:2), the GM of HT and βCD (molar ratio = 1:1), and the GM of HT and γCD (molar ratio = 1:1) had MICs of 10 μg/mL with respect to C. albicans and 10 μg/mL with respect to A. brasiliensis. Results of the antifungal test indicated that GMs had a lower MIC than that of HT alone, so the antifungal activity of HT with respect to C. albicans and A. brasiliensis was enhanced. Nevertheless, a decrease in the MIC like that seen in antibacterial testing was not evident. Essential oil components are reported to exhibit antifungal action by inhibiting the growth of hyphae. Moreover, essential oil components are known to cause the cell wall to lose rigidity and integrity and the loss of cytoplasmic components, resulting in cell death. Accordingly, differences in HT’s mechanism of action with respect to bacteria and fungi or differences in the effects of CD due to structural differences in bacteria and fungi may have an effect. Essential oil components are reported to exhibit different types of activity on bacteria and fungi. This is attributable to structural differences in their cell walls. Essential oil components are known to exhibit antifungal action by affecting the functioning of mitochondria since they inhibit the action of mitochondrial dehydrogenases involved in fungal ATP synthesis. In addition, essential oils are known to inhibit ergosterol synthesis by fungi. Ergosterol serves to stabilize the fungal cell membrane and is not found in bacteria. Therefore, it is possible that the fluctuation of the HT MIC value in the HT/CD complex is due to a mechanism different from that of bacteria.

Differences in HT’s mechanism of action on bacteria and fungi as mentioned above may help to sustain the activity of HT when HT/CD complexes are formed. In the future, detailed evaluation of antibacterial activity using fungi is required.

2.3. Time-Kill Assay. A time-kill assay is used to assess a substance’s typical bactericidal and bacteriostatic actions with respect to a bacterium over time. Thus, a time-kill assay was performed to assess the time-dependent antibacterial action of HT and HT/CD inclusion complexes on E. coli, a Gram-negative bacterium. In this study, E. coli was selected as the target strain for the time-kill assay test. E. coli, a Gram-negative bacterium, is one of the major species of bacteria present in the environment. Therefore, E. coli inhabits the digestive tract from normal times and causes intestinal infectious diseases. In addition, E. coli is a cause of urinary tract infections and is an important bacterium in clinical susceptibility testing.

Some viable bacterial cells were noted in the control group not treated with HT immediately after the start of the assay (0 h). In contrast, HT alone (320 μg/mL) caused a marked decrease in the viable bacterial count at 3 h in comparison to the count in the control group, and the detection limit was reached at 6 h (Figure 2). This presumably indicates that HT

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**Table 2. Antifungal Activity of HT/CD Systems**

| samples | MIC (μg/mL) | C. alb | A. bra |
|---------|-------------|--------|--------|
| CDs     | n.d.        | n.d.   |        |
| HT intact | 20          | 20     |        |
| HT ground | 20          | 20     |        |
| PM (HT/αCD = 1:2) | 20 | 20 |        |
| PM (HT/βCD = 1:1) | 20 | 20 |        |
| PM (HT/γCD = 1:1) | 20 | 20 |        |
| GM (HT/αCD = 1:2) | 10 | 10 |        |
| GM (HT/βCD = 1:1) | 10 | 10 |        |
| GM (HT/γCD = 1:1) | 10 | 10 |        |

*C. alb: Candida albicans, A. bra: Aspergillus brasiliensis. *All MIC values are converted into HT concentration. *n.d.: not determined.

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**Figure 2. Time-kill assay for E. coli in the presence of intact HT.**

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Some viable bacterial cells were noted in the control group not treated with HT immediately after the start of the assay (0 h). In contrast, HT alone (320 μg/mL) caused a marked decrease in the viable bacterial count at 3 h in comparison to the count in the control group, and the detection limit was reached at 6 h (Figure 2). This presumably indicates that HT

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HT and βCD (molar ratio = 1:1) gradually inhibited growth at 3 h compared to HT alone. The GM of HT and βCD (molar ratio = 1:1) caused the colony count to decrease over time for up to 12 h, so the release of HT was presumably inhibited as a result of inclusion in a CD.

HT/CD complexes inhibited the growth of E. coli in a short amount of time compared to HT alone. A factor for this is presumably the faster rate at which HT reached the surface of bacterial cells as a result of its inclusion in a CD. In the current study, PMs behaved like HT alone, so this phenomenon should be specific to inclusion complex formation. According to Kogawa et al., a drug from a CD complex is delivered to the lipophilic bacterial membrane, causing the drug to exhibit its pharmacological action. In the current study, HT/CD complexes exhibited faster antibacterial action than HT alone; one factor for this is presumably the fact that CD transports HT to the surface of bacterial cells. In addition, HT exhibited differing antibacterial actions in αCD, βCD, or γCD complexes, so differences in a CD’s affinity for a bacterial cell membrane presumably led to differences in the ability of HT to reach the cell membrane.

2.4. Measurement of Membrane Potential. Changes in the membrane potential of a bacterium as a result of the addition of CDs were assessed to fully elucidate the mechanism of antimicrobial action of HT/CD inclusion complexes. The membrane potential plays an important role in cytophysiological processes. Moreover, the membrane potential reflects the integrity and energy state of the cell membrane and the cell’s survival rate. Typically, loss of the membrane potential is known to occur in conjunction with an increase in membrane penetration. Thus, HT and CD were tested using DiBAC4(3), a voltage-sensitive dye, to determine whether they affected the membrane potential of a bacterium. The test bacterium used was E. coli, which is a Gram-negative bacterium. Testing was performed using flow cytometry (FCM).

The assay of the membrane potential revealed that DiBAC4(3) had a fluorescence intensity in the range of 0–100 in the untreated sample (negative control) (Figure 4).

Thus, the untreated sample had only viable bacterial cells, so the fluorescence intensity of viable bacterial cells was close to 0, indicating a low level of fluorescence. In contrast, DiBAC4(3) had a fluorescence intensity in the range of 101–102 in the heated sample (control). Thus, the heated sample had only dead bacterial cells, so the fluorescence intensity of dead bacterial cells was close to 102, indicating a high level of fluorescence. CCCP is an uncoupling agent that is used to depolarize the cell membrane. The assay was performed with CCCP as a positive control. Results were similar to those for the heated sample. DiBAC4(3) is a slow-acting voltage-sensitive probe. Typically, DiBAC4(3) enters depolarized cells, where it binds with intracellular proteins or the cell membrane, and then strongly fluoresces. An increase in depolarization indicates further inflow of the anionic dye and increased fluorescence. Thus, the dye weakly fluoresced in polarized viable bacterial cells and strongly fluoresced in depolarized dead bacterial cells.

Fluorescence increased with αCD, βCD, and γCD when some bacterial cells were viable or dead. Thus, the addition of CD may have disrupted the membrane potential of E. coli. In contrast, HT alone did not cause marked changes in the electric potential at a concentration of 20 μg/mL. In addition, the membrane potential shifted at a concentration of 80 μg/mL. These findings suggest that HT affected the membrane potential of the bacterium in a dose-dependent manner. However, only slight changes in the membrane potential were noted, so presumably, a certain concentration of HT must be present to cause the membrane potential to change. Therefore, it was speculated that the HT/CD complex was caused by the change in bacterial membrane potential caused by CD, and the

Figure 3. (a) Time-kill curves for E. coli in the presence of HT/CD systems (HT concentration: 80 μg/mL). (b) Time-kill curves for E. coli in the presence of HT/CD systems (HT concentration: 320 μg/mL).

Figure 4. Flow cytometric analysis of E. coli with HT and CDs. (A) Control, (B) negative control, (C) positive control (CCCP), (D) αCD, (E) βCD, (F) γCD, (G) intact HT (20 μg/mL), and (H) intact HT (80 μg/mL).
effective antibacterial action of HT was expressed by increasing the membrane permeability of HT.

2.5. Flow Cytometric Analysis of Bacterial Cells. The LIVE/DEAD BacLight Bacterial Viability Kit was used to assess the cell membrane damage caused by HT to determine whether or not HT and CD affected the bacterial cell membrane. BacLight simultaneously stains samples with two nuclear staining reagents (SYTO9: green and PI: red) with different degrees of cell membrane penetration; this allows differentiation of viable cells (undamaged) and dead cells (damaged). This property allows a determination of whether bacterial cells are viable or dead based on whether they are damaged or not, and whether HT has caused membrane damage or not was determined based on those results. The test bacterium used was E. coli, which is a Gram-negative bacterium.

HT alone increased the fluorescence intensity of PI (indicating the dead bacterial count) in a dose-dependent manner (Figure 5). The viable bacterial count tended not to increase again over time, so HT presumably had irreversible antibacterial action, i.e., bactericidal action, on E. coli. Thus, HT may exhibit antibacterial action by damaging the cell membrane.

When CD alone was added, in contrast, the viable bacterial count was roughly similar to that in the control at 3, 6, and 24 h. CD alone presumably did not damage the bacterial cell membrane (a physical barrier) (Figure 6). This finding indicates that CD alone does not affect the bacterial cell membrane.

A number of viable bacterial cells were exposed to HT for 24 h when the GM of HT and αCD (molar ratio = 1:2) was added (Figure 7). E. coli were lysed as a result of exposure to HT, so cells did not fluoresce since they were not stained by the nuclear staining reagents. Dead bacterial cells were not noted, and viable bacterial cells may have increased in relative terms. Adding the GM of HT and βCD (molar ratio = 1:1)
resulted in delayed bactericidal action of HT compared to that of HT alone at every concentration. This may indicate that the release of HT was inhibited as a result of its inclusion in βCD. Adding the GM of HT and γCD (molar ratio = 1:1) resulted in the death of about 61% of the bacterial cells at 3 h and the death of about 75% of the bacterial cells 24 h later. These findings presumably indicate that the GM of HT and γCD (molar ratio = 1:1) exhibited antibacterial action by causing the rapid release of HT.

These findings indicate that inclusion of HT in a CD is key to HT/CD complexes reaching the cell membrane. This inclusion enhances HT’s penetration of the bacterial cell membrane (a barrier) and may increase the antimicrobial activity of HT. HT at a low concentration behaved differently than HT alone, so the antibacterial action of HT may be efficiently displayed as a result of the formation of an inclusion complex. Differences in the extent of HT’s antibacterial activity were evident in HT/CD complexes formed by different CDs. This indicates that inclusion in a CD contributes to the structural stability of HT. A previous assessment of the physical properties of HT/CD complexes revealed different forms of inclusion depending on the CD used. This causes changes in the structural state of the HT molecule in a CD and may be reflected in differences in HT’s potency against different bacteria. The main Gram-negative bacteria that pose a problem in medical infection control are E. coli and P. aeruginosa. In particular, the HT/CD complex can improve the antibacterial activity of E. coli as a Gram-negative bacterium and elucidate the foundation of its antibacterial mechanism; it will be a useful subject for research and development as a future clinical application.

3. CONCLUSIONS
The current results revealed that HT and HT/CD complexes are active against bacteria and fungi. HT irreversibly inhibited the growth of microbes in a short amount of time. In each of the antimicrobial tests, there was no decrease in antimicrobial activity even if HT was included in a CD, and HT/CD complexes exhibited activity equivalent to or greater than that of HT alone. The mechanism of action may be due to the fact that HT/CD complexation disturbed the cell membrane potential of bacteria and improved the membrane permeability of HT. Thus, including an antimicrobial component in CD should lead to appropriate control of the rate of drug release and efficient use of antimicrobial activity.

4. MATERIALS AND METHODS
4.1. Materials. 4.1.1. Chemicals. HT from FujiFilm Wako Pure Chemical Corporation was used as it is. αCD, βCD, and γCD were kindly provided by CycloChem and stored in a humidity-controlled environment at a temperature of 40 °C and an RH of 82%. Other reagents from FujiFilm Wako Pure Chemical Corporation were of special reagent grade.

4.1.2. Preparation of Physical Mixtures and Ground Mixtures. HT and αCD were weighed to obtain a molar ratio of 1:2, and HT and βCD and HT and γCD were weighed to obtain a molar ratio of 1:1. HT was mixed with αCD, βCD, or γCD for 1 min using a Vortex Mixer to yield three physical mixtures (PMs). Each PM (1 g) was placed on an aluminum pan and then co-ground with a vibrating rod mill (TI-500ET, CMT) for 60 min to serve as a ground mixture (GM).

4.2. Methods. 4.2.1. Antibacterial Testing. The minimum inhibitory concentration (MIC) of HT as a result of agar dilution was based on Standard M7-A5 of the Clinical and Laboratory Standards Institute (CLSI) M7-A5; this determination was in accordance with the methods described by Takeda et al.34,35 Four bacteria were tested: two Gram-positive bacteria (Bacillus subtilis NBRC3134 and Staphylococcus aureus JCM2413) and two Gram-negative bacteria (Escherichia coli JCM5491 and Pseudomonas aeruginosa JCM6119). Mueller-Hinton II Agar (MHA, BD) was used as the test medium. All of the samples were added to 100 mL of MHA so that the concentration of HT would be 320 μg/mL, and each mixture was carefully stirred without producing bubbles. MHA containing HT was serially diluted twofold with MHA to prepare a medium with a twofold dilution of HT. The prepared test medium was poured into a Petri dish, where it solidified. A suspension of each test bacterium was prepared with 2.0 × 10⁶ CFU/mL of each bacterium. Each test medium was inoculated with 5 μL of that suspension and allowed to dry. Test plates were cultured for 24 h in an incubator at 37 °C. Plates were visually inspected for colony formation after culturing, and the MICs of HT and HT/CD complexes with respect to each species were determined.

4.2.2. Antifungal Testing. The antifungal action of HT was assessed using the agar dilution method to calculate the MIC of HT with respect to fungi. Two fungi were tested: Candida albicans and Aspergillus brasiliensis. HT and HT/CD complexes were prepared with sterile water and diluted with RPMI1640 medium (containing glucoseamine). Each solution was buffered to a pH of 7.0 using 0.165 M 3-(N-morpholino)propanesulfonic acid (MOPS) buffer. A sample was added so that the concentration of HT would be 320 μg/mL, and the mixture was carefully stirred without producing bubbles. RPMI1640 medium containing HT was serially diluted twofold with RPMI1640 medium to prepare medium with a twofold dilution of HT. A 3% agar solution was added to the prepared test medium; the mixture was fully stirred and then poured into a Petri dish, where it solidified. A suspension of each test bacterium was prepared with 2.0 × 10⁶ CFU/mL of each bacterium. Each test plate was inoculated with 5 μL of that suspension and allowed to dry. Test plates were cultured for 48–72 h in an incubator at 37 °C. Plates were visually inspected for colony formation after culturing, and the MICs of HT and HT/CD complexes with respect to each species were determined.

4.2.3. Time-Kill Assay. A time-kill assay was performed according to the methods of Baltch et al. based on CLSI standards.36,37 Sterile PBS was sprayed on a test bacterium that had been cultured on an agar slant to prepare a stock suspension of the test bacterium. The stock suspension of the test bacterium was added to Mueller-Hinton II Broth (MHB) until the final concentration reached 1.0 × 10⁶ CFU/mL. Afterward, the suspension was shaken with a rotary shaker (NR-3, TITEC) during culturing at 37 °C until its OD at 600 nm >0.1 according to an absorption spectrometer. The suspension of the test bacterium was diluted with the test solution (distilled water) until its final concentration reached 1.5 × 10⁵ CFU/mL. A sample was added, and the suspension was shaken (150 rpm) again at 25 °C. The culture solution was collected immediately prior to adding a sample (0 h) and 3, 6, 12, and 24 h after a sample was added to serve as the stock solution. The stock solution was applied to Tryplicase Soy Agar medium (TSA medium, BD), which was cultured at 37 °C.
4.2.4. Measurement of Membrane Potential. A membrane potential assay was performed using DiBAC$_4$(3) (bis(1,3-dibutylbarbituric acid)trimethine oxonol), and sodium salt (Dojindo Laboratories) as an intracellular fluorescent probe. The test bacterium used was a Gram-negative bacterium (E. coli JCM5491). Carbonyl cyanide m-chlorophenylhydrazone (CCCP) does not affect the electric potential of a cell membrane, so it was used as a positive control. The cultured bacterial solution was sterilized in an autoclave at 100 °C for 30 min to serve as the negative control. A bacterial solution was prepared by shaking (130 rpm) a stock suspension of the test bacterium during culturing at 37 °C for about 4 h until its final concentration reached 5.0 × 10$^6$ CFU/mL. After the bacterium and a sample were added to the test solution, the mixture was shaken again (150 rpm) at 25 °C. The culture solution was collected 1 h after a sample was added to serve as the stock solution. The collected culture solution was centrifuged. The supernatant was suctioned off and allowed to react with the DiBAC$_4$(3) reagent. Fluorescence intensity was measured with the EPICS XL Flow Cytometer (Beckman Coulter).

4.2.5. Flow Cytometric Analysis of Bacterial Cells (Assessment of Membrane-Damaging Action). A membrane damage assay was performed using the LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Inc.). The test bacterium used was a Gram-negative bacterium (E. coli JCM5491). A stock suspension of the test bacterium was shaken (130 rpm) during culturing at 37 °C for about 4 h until its final concentration reached 5.0 × 10$^6$ CFU/mL. After the bacterium and a sample were added to the test solution, the mixture was shaken again (150 rpm) at 25 °C. A sample was added, and the culture solution was collected 6 and 24 h later to serve as the stock solution. The collected culture solution was centrifuged. The supernatant was suctioned off and allowed to react with the BacLight reagent. After incubation at 37 °C for 15 min, live and dead bacterial cells were counted with the EPICS XL Flow Cytometer (Beckman Coulter).

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Notes
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