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Riccardin C derivatives cause cell leakage in *Staphylococcus aureus*

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major problem in clinical settings, and because it is resistant to most antimicrobial agents, MRSA infections are difficult to treat. We previously reported that synthetic macrocyclic bis(bibenzyl) derivatives, which were originally discovered in liverworts, had anti-MRSA activity. However, the action mechanism responsible was unclear. In the present study, we elucidated the action mechanism of macrocyclic bis(bibenzyl) RC-112 and its partial structure, IDPO-9 (2-phenoxyphenol). Survival experiments demonstrated that RC-112 had a bactericidal effect on MRSA, whereas IDPO-9 had bacteriostatic effects. IDPO-9-resistant mutants exhibited cross-resistance to triclosan, but not to RC-112. The mutation was identified in the *fabI*, enoyl-acyl carrier protein reductase gene, a target of triclosan. We have not yet isolated the RC-112-resistant mutant. On the other hand, the addition of RC-112, unlike IDPO-9, caused the inflow of ethidium and propidium into *S. aureus* cells. RC-112-dependent ethidium outflow was observed in ethidium-loaded *S. aureus* cells. Transmission electron microscopy also revealed that *S. aureus* cells treated with RC-112 had intracellular lamellar mesosomal-like structures. Intracellular Na^+ and K^+ concentrations were significantly changed by the RC-112 treatment. These results indicated that RC-112 increased membrane permeability to ethidium, propidium, Na^+, and K^+, and also that the action mechanism of IDPO-9 was different from those of the other compounds.

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1. Introduction

Multidrug-resistant bacteria are currently a major problem in clinical settings. Methicillin-resistant *Staphylococcus aureus* (MRSA), in particular, is one of the main causes of nosocomial infection. *S. aureus* has been shown to cause suppuration, food poisoning, exfoliative dermatitis, toxic shock syndrome, urinary infection, and sepsis. More severe symptoms, such as pneumonia and meningitis, have been reported in immunocompromised hosts, including the elderly. *S. aureus* is known to form biofilms on catheters and joint prostheses, and also causes persistent infection. Therefore, antimicrobial therapies are needed to effectively treat patients infected with *S. aureus*.

Vancomycin, teicoplanin, linezolid, arbekacin, and daptomycin are typically used to treat MRSA infections in our country. Previous studies demonstrated that glycopeptides, including vancomycin and teicoplanin, inhibited cell wall synthesis [1,2], linezolid and arbekacin inhibited protein synthesis [3,4], and daptomycin disrupted multiple aspects of bacterial cell membrane function [5]. Although the effectiveness and usefulness of these drugs in the treatment of MRSA infections have been confirmed, resistant mutants to these drugs have been isolated. Therefore, the discovery and development of new antimicrobial agents are important.

We previously reported that riccardin C exhibited anti-MRSA activity [6,7]. Riccardin C, a macrocyclic bis (bibenzyl) compound, was originally discovered in liverworts [8], and exhibits cyclooxygenase (COX)-inhibitory [9], HIV-1 reverse transcriptase-inhibitory [10], antifungal [10], the nuclear receptor family transcription factors Liver X Receptor (LXR)-modulating [11], and anti-cancer [12] activities. However, its action mechanism as an antibacterial agent remains poorly understood.

We here investigated the antibacterial mechanisms of riccardin C derivatives and the partial structure of riccardin C. The anti-MRSA effects of riccardin C derivatives were bactericidal. On the other hand, the anti-MRSA activity of IDPO-9, a 2-hydroxyl phenyl ether that is the partial structure of riccardin C, was weaker than that of the riccardin C derivatives. Furthermore, the anti-MRSA effects of IDPO-9 were

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CFU, colony forming units; MIC, minimal inhibitory concentration; MRSA, methicillin-resistant *Staphylococcus aureus*; OD, optical density.

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bacteriostatic. Our results indicated that the antibacterial mechanism of riccardin C derivatives was the induction of cell leakage while that of IDPO-9 was the inhibition of FabI.

2. Materials and methods

2.1. Bacterial strains and growth conditions

S. aureus N315 (MRSA strain) is an MRSA strain in which the genome project has been completed [13]. *S. aureus* and *Bacillus subtilis* 168 cells were grown in Nutrient Broth Nissui (N broth) (Nissui Pharmaceutical Co., Ltd) at 37 °C with shaking. *Enterococcus faecalis* NCTC12201 cells were grown in Brain Heart Infusion broth (Becton, Dickinson and Company) at 37 °C without shaking. Gram-negative bacteria (*Escherichia coli* TG1, *Pseudomonas aeruginosa* PAO1, and *Vibrio parahaemolyticus* AQ3334) were cultured in L-broth. Cell growth was monitored at a wavelength of 650 nm with a spectrophotometer.

2.2. Antimicrobial agents and chemicals

Riccardin C derivatives were synthesized as described previously (Fig. 1) [6,7].

2.3. Drug susceptibility testing

Minimal inhibitory concentrations (MICs) were determined using the broth microdilution method according to the recommendations of the Japanese Society of Chemotherapy. Briefly, MICs for various drugs were determined in Mueller–Hinton (MH) broth (Becton, Dickinson and Company) containing different drugs at various concentrations as reported previously [14]. Cells were incubated in the medium at 37 °C for 24 h, and growth was assessed by visual inspection.

2.4. Survival assay

S. aureus cells were cultured in N broth at 37 °C until $OD_{650} = 0.7$. Riccardin C derivatives were added at 1/4, 1, or 4 times the MIC. After being incubated at 37 °C, a 50 μL portion was collected at each time point, and the serially diluted culture was plated on the N broth agar. Plates were incubated at 37 °C for 16–24 h and the number of colonies was then counted to calculate the colony forming unit (CFU).

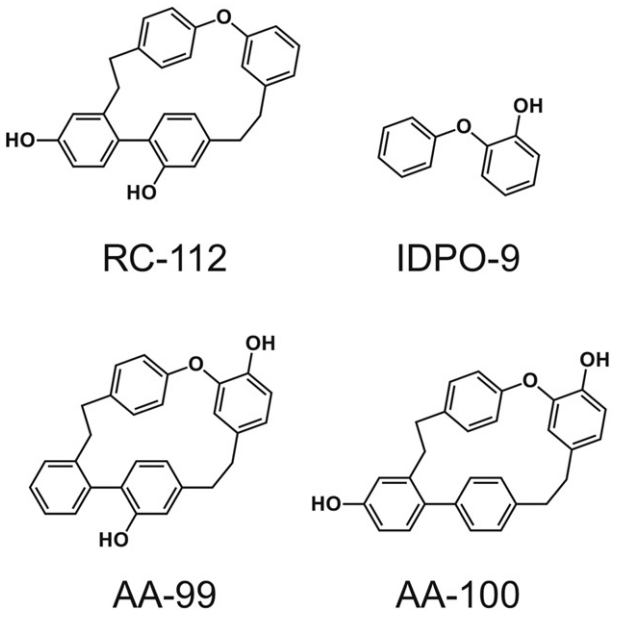

Fig. 1. Chemical structure of compounds.

2.5. Ethidium inflow assay

Cells were harvested at the late exponential phase ($OD_{650} = 0.7$) of growth and washed twice with chilled N broth. They were then centrifuged, washed, and resuspended in chilled N broth ($OD_{650} = 0.3$). The cells were incubated at 37 °C and 25 μM ethidium bromide was added. The inflow of ethidium was evaluated as an increase in fluorescence intensity after the addition of riccardin C derivatives (excitation 530 nm and emission 600 nm).

2.6. Propidium inflow assay

Cells were harvested at the late exponential phase ($OD_{650} = 0.7$) of growth and washed twice with chilled N broth. They were then centrifuged, washed, and resuspended in chilled N broth ($OD_{650} = 0.4$). The cells were incubated at 37 °C and 10 μg/ml propidium iodide was added. The inflow of propidium was evaluated as an increase in fluorescence intensity after the addition of riccardin C derivatives (excitation 535 nm and emission 617 nm).

2.7. Ethidium outflow assay

Cells were harvested at the late exponential phase ($OD_{650} = 0.7$) of growth and washed twice with 20 mM HEPES–NaOH (pH 7.0). Cells were suspended in the HEPES–NaOH buffer containing 20 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and 40 μM ethidium bromide, adjusted to adjust the $OD_{650} = 0.4$, followed by incubation at 37 °C for 30 min. The cells were centrifuged, washed, and resuspended in the HEPES–NaOH buffer to make $OD_{650} = 0.4$. The outflow of ethidium was evaluated as a decrease in fluorescence intensity (excitation 530 nm and emission 600 nm).

2.8. Scanning electron microscopy

S. aureus or *B. subtilis* cells were cultured in N-broth and harvested at the late exponential phase ($OD_{650} = 0.7$) of growth. After washing, the cells were treated with AA-99 (8 μg/ml) and DMSO (0.8%) for 3 h at 37 °C. Cells were dropped on a glass fiber filter (GF-75 (ADVANTEC)) coated with 0.5% agar N-broth. The filters were fixed in a mixture of 2% glutaraldehyde and 2% paraformaldehyde overnight at 37 °C [15]. After washing with buffer (0.1 M phosphate buffer (pH 7.2)), cells were dehydrated by a graded series of ethanol. Finally, water in cells was replaced with t-butyl alcohol. These samples were freeze-dried and sputter coated with OsO_4, and examined under a scanning electron microscope. These experiments were performed at the Central Research Laboratory, Okayama University Medical School.

2.9. Transmission electron microscopy

S. aureus cells were cultured in N-broth and harvested at the late exponential phase ($OD_{650} = 0.7$) of growth. After washing, the cells were treated with RC-112 (8 μg/ml) and DMSO for 90 min at 37 °C. Samples for the electron micrograph were prepared as described previously [16]. The pellets of treated cells were fixed in a mixture of 2.5% glutaraldehyde and 1% OsO_4 for 2 h on ice. After washing with buffer (0.1 M phosphate buffer (pH 7.4)), the block was prepared with agarose. The specimens were dehydrated by ethanol, replaced with propylene oxide, and embedded in TAAB Low Viscosity Resin. Ultrathin sections (80 nm each) were cut on an ultramicrotome (Leica EM UC7), double staining was performed with a saturated solution of uranyl acetate and Reynolds' lead citrate, and specimens were then examined under an H-7650 transmission electron microscope (Hitachi, Japan). These experiments were performed at the Central Research Laboratory, Okayama University Medical School.

2.10. Measurement of intracellular ion concentrations

Cells were cultured in N broth at 37 °C until $OD_{650} = 0.7$. After the addition of various agents, cells were incubated at 37 °C for 90 min. Each sample was centrifuged (15,000 rpm at 4 °C for 3 min) to separate the cells and the cell-free supernatant. Separated cells were freeze-dried and acid-digested with nitric acid (HNO_3) at 95 °C [17]. Acid-digested samples were diluted with MilliQ and subjected to an inductively coupled plasma optical emission spectrometry (ICP-OES) analysis. ICP-OES was performed by VISTA-PRO (Seiko Instruments, Inc.).

3. Results

3.1. Effect of the riccardin C derivatives on survival of S. aureus

We previously reported that the riccardin C derivatives, RC-112, AA-99, and AA-100, as well as the partial structure of riccardin C, IDPO-9, exhibited anti-MRSA activities (Fig. 1) [6,7]. To investigate the action mechanism of riccardin C derivatives, we first performed a survival assay for *S. aureus* N315.

RC-112, AA-99, and AA-100 exhibited strong anti-MRSA activities (Table 1) [6,7]. When RC-112 was added at the concentration of the MIC, the number of viable cells was not decreased or significantly different from that of the control (no addition). However, a marked decrease was observed in the number of viable cells when RC-112 was added at a concentration that was 4 times the MIC (Fig. 2A). Regarding AA-99 and AA-100, the number of viable cells was reduced by at least two orders of magnitude at the concentration of the MIC, and four orders at 4 times the MIC (Fig. 2C, D). These results indicated that these three compounds had bactericidal effects on *S. aureus* N315.

On the other hand, a decrease in the number of viable cells was not observed when IDPO-9, the partial structure of riccardin, was added (Fig. 2B), even at 4 times the MIC. These results suggested that the action mechanisms of IDPO-9 and the other compounds differed from each other.

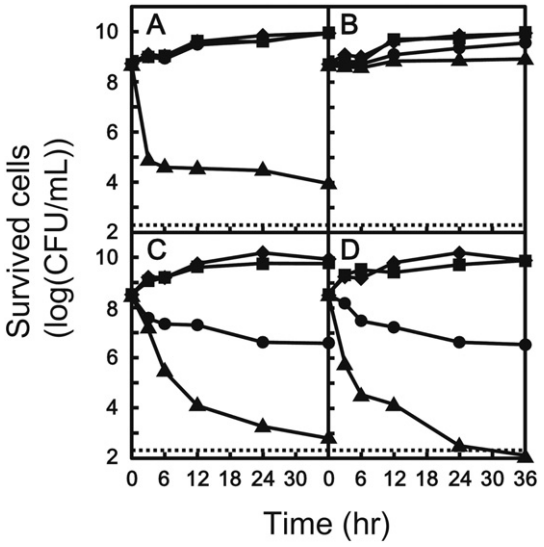

Fig. 2. Survival assay for the riccardin C derivatives against *S. aureus* N315. Mid-log-phase organisms (10^8 CFU/ml) were incubated with various concentrations of the riccardin C derivatives in DMSO, RC-112 (A), IDPO-9 (B), AA-99 (C), and AA-100 (D). Control (diamond): DMSO, concentrations that were a quarter times the MIC (square): IDPO-9 (2 μg/ml), RC-112 (0.25 μg/ml), AA-99, and AA-100 (0.5 μg/ml), concentrations that were equivalent with the MIC (circle): IDPO-9 (8 μg/ml), RC-112 (1 μg/ml), AA-99, and AA-100 (2 μg/ml), concentrations that were four times the MIC (triangle): IDPO-9 (32 μg/ml), RC-112 (4 μg/ml), AA-99, and AA-100 (8 μg/ml). The dashed line indicated the detection limit of this experiment. Three independent experiments were performed, and representative data were shown.

3.2. Isolation of resistant mutants

To investigate antibacterial mechanisms, we attempted to isolate RC-112- and IDPO-9-resistant mutants from *S. aureus* N315. To isolate each resistant mutant, N315 cells were spread on an N agar plate containing 8 μg/ml RC-112 or 32 μg/ml IDPO-9 (4 times MICs). We isolated the IDPO-9-resistant mutant at a frequency of approximately 10^{-10}, but have not yet isolated any RC-112-resistant mutants. We named the IDPO-resistant mutant HDR32.

The IDPO-9-resistant mutant HDR32 had a higher MIC to IDPO-9 (>128 μg/ml) and triclosan (2 μg/ml) (Table 1). However, the MIC of HDR32 to most other drugs, including arbekacin, vancomycin, tetracycline, and ethidium bromide was identical to that of the parent strain N315. HDR32 did not have elevated MICs for the riccardin C derivatives such as RC-112, AA-99, and AA-100. IDPO-9 was shown to have a similar chemical structure to triclosan, which inhibits FabI, an enoyl-acyl carrier protein reductase [18,19]. Therefore, we attempted to isolate triclosan-resistant mutants. *S. aureus* N315 cells were spread on an N agar plate containing 0.5 μg/ml triclosan (8 times the MIC). The triclosan-resistant mutants TRR1 and TRR2 showed elevated MICs for IDPO-9 and triclosan, but not for the other riccardin C derivatives and antibacterial agents. These results also implied that riccardin C derivatives may have different antibacterial mechanisms from that of IDPO-9. IDPO-9 and triclosan were found to share similar antibacterial mechanisms, such as the inhibition of FabI.

3.3. Identification of a mutation in fabI in triclosan-resistant mutants and the IDPO-9 mutant

IDPO-9 and triclosan-resistant mutants exhibited cross-resistance to each other. Therefore, these compounds may have similar antibacterial mechanisms. Since a single amino acid change in FabI or the overexpression of FabI was previously detected in the triclosan-resistant mutant of *S. aureus* [20,21], we sequenced the *fabI* coding regions and promoter regions of triclosan-resistant mutants, TRR1 and TRR2, as well as the IDPO-9 resistant mutant, HDR32. A single mutation was identified in the *fabI* coding region of all mutants (Table 2). The predicted FabI amino acid change in both TRR1 and TRR2 was Ala 95 to Val relative to the sequence of the wild type parent strain N315. The predicted FabI amino acid change of HDR32 was Phe 204 to Ser.

The X-ray crystal structure of triclosan bound to the FabI of *S. aureus* N315 was reported previously [22] (Fig. 3A). We predicted the structure

Table 1
MICs of the Riccardin C derivatives, ethidium bromide, and triclosan.

	S. aureus				*B. subtilis*	*E. faecalis*	*E. coli*	*P. aeruginosa*	*V. parahaemolyticus*
	N315	HDR32	TRR1	TRR2	168	NCTC12201	TG1	PAO1	AQ3334
RC-112	1	1	1	1	4	4	>128	>128	>128
AA-99	2	2	2	2	2	4	>128	>128	>128
AA-100	2	1	2	2	2	2	>128	>128	>128
IDPO-9	8	>128	>128	>128	>128	>128	32	>128	128
Triclosan	0.0625	2	>2	>2	2	2	<1	>8	4
Ethidium Br	8	8	4	4	n.d.	n.d.	n.d.	n.d.	n.d.

n.d.; not determined.

Table 2
Mutation in FabI in the *S. aureus* mutants.

Strain	Nucleotide change	Amino acid change
HDR32	611T to C	F204S
TRR1	284C to T	A95V
TRR2	284C to T	A95V

of the mutant FabI based on the alignment of wild type FabI using SWISS-MODEL [23–27] (Fig. 3B, C and D). The FabI mutation in TRR1 and TRR2 induced structural changes near the triclosan binding site, such that Val 95 could interfere with the predicted binding of triclosan. The FabI mutation in HDR32 changed the triclosan-binding hydrophobic pocket. Since Ser is known to be a more hydrophilic amino acid than Phe, this substitution may decrease hydrophobic interactions between triclosan and the hydrophobic pocket.

When the tertiary structures of the riccardin C derivatives were compared with triclosan and IDPO-9, riccardin C derivatives were found to have bulkier structures. It was also difficult to impose riccardin C derivatives on the simulated structure of FabI (data not shown). These results indicated that riccardin C derivatives, except for IDPO-9, had no effect on FabI, and may have other targets.

3.4. Effects of the riccardin C derivatives on ethidium inflow and outflow

The riccardin C derivatives, except for IDPO-9, exhibited bactericidal effects. Loss of membrane function is known to be one of the bactericidal mechanisms. Therefore, we determined whether riccardin C derivatives influenced the membrane function of several bacteria. If membrane permeability increased due to the effects of the riccardin C derivatives, some molecules should have leaked into the cells according to the concentration gradient. The MICs for the riccardin C derivatives were lower against Gram-positive bacteria (*S. aureus*, *E. faecalis* and *B. subtilis*) than those against Gram-negative bacteria (*E. coli*, *P. aeruginosa* and *V. parahaemolyticus*) (Table 1). We then measured the inflow of ethidium into Gram-positive bacterial cells.

When RC-112 was added to energized *S. aureus* N315 cells, the fluorescence of ethidium immediately increased (Fig. 4A). A similar increase was observed when CCCP was added as the control, which indicated that the intracellular ethidium concentration increased. Two possibilities were considered for the increase observed in intracellular ethidium. One was the increase of membrane permeability, and the other was the inhibition of multidrug efflux pumps that extrude ethidium. We next investigated the effects of RC-112 on the outflow of ethidium from ethidium-loaded *S. aureus* cells. The addition of RC-112 caused a rapid decrease in ethidium fluorescence in a RC-112 concentration-dependent manner, similar to the detergent SDS (Fig. 4B). If RC-112 inhibited efflux pumps, we would not be able to detect any changes in fluorescence in the ethidium outflow assay. These results strongly suggested that RC-112 increased the permeability of the cytoplasmic membrane of *S. aureus*. The addition of DMSO as a negative control did not induce any significant changes in ethidium fluorescence intensity.

A previous study reported that triclosan caused the leakage of intracellular contents when added at a concentration higher than the MIC [28]. However, the addition of IDPO-9 did not cause the inflow of ethidium, even at a concentration that was ten times the MIC (Fig. 4A).

3.5. Effects of the riccardin C derivatives on propidium inflow

Since ethidium is known to be exported via multidrug efflux pumps, we could not exclude the possibility that some efflux pumps may be damaged by RC-112. Therefore, we used propidium iodide, which stains dead cells, because it is normally a membrane-impermeable fluorescence dye but can enter cells when their membranes are damaged. When RC-112 was added, propidium fluorescence intensity rapidly increased (Fig. 5). This result indicated that riccardin C derivatives cause cell leakage.

3.6. Morphological changes in S. aureus N315 treated with RC-112

As described above, riccardin C derivatives may cause membrane cell leakage, which may, in turn, induce morphological changes in bacteria. However, using light microscopy, we could not observe morphological changes in *S. aureus* N315 or *B. subtilis* 168 cells treated with RC-112 (data not shown). We then used scanning electron microscopy (SEM) and transmission electron microscopy (TEM). In *B. subtilis*, we found significant changes. After a 3 h incubation with 8 μg/ml AA-99, the membrane became corrugated and/or partially swelled (Fig. 6). It

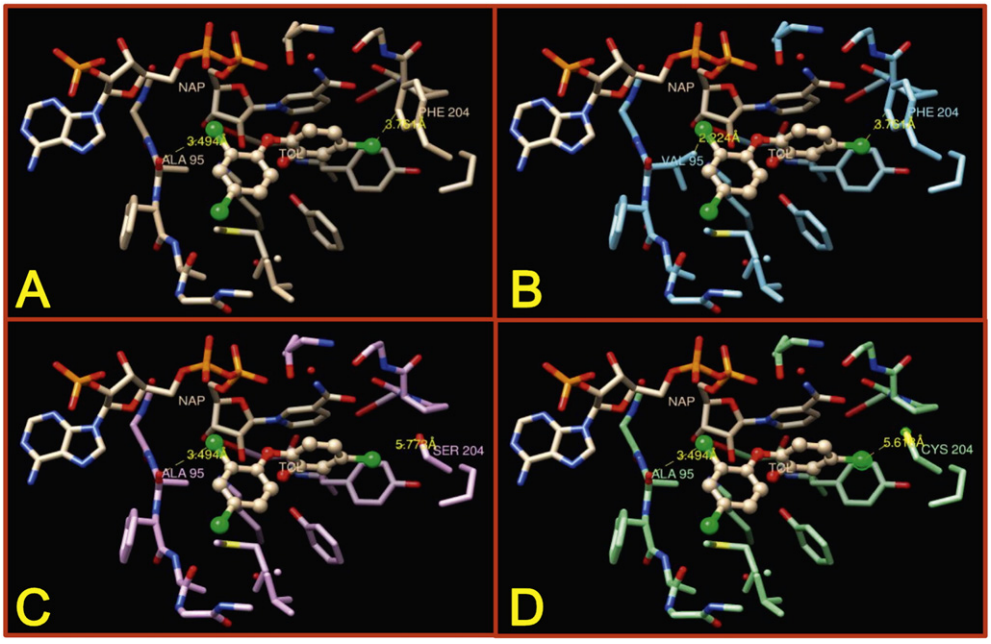

Fig. 3. Structure of saFabI complexed with NAD^+ and triclosan. The X-ray crystal structure of triclosan bound to wild-type *S. aureus* FabI (PDB entry: 4ALIA) (A) and a simulated model of triclosan bound to A95V (B) and to F204S (C) and F204C (D) (21) saFabI. These models were made using the molecular modeling system UCSF chimera.

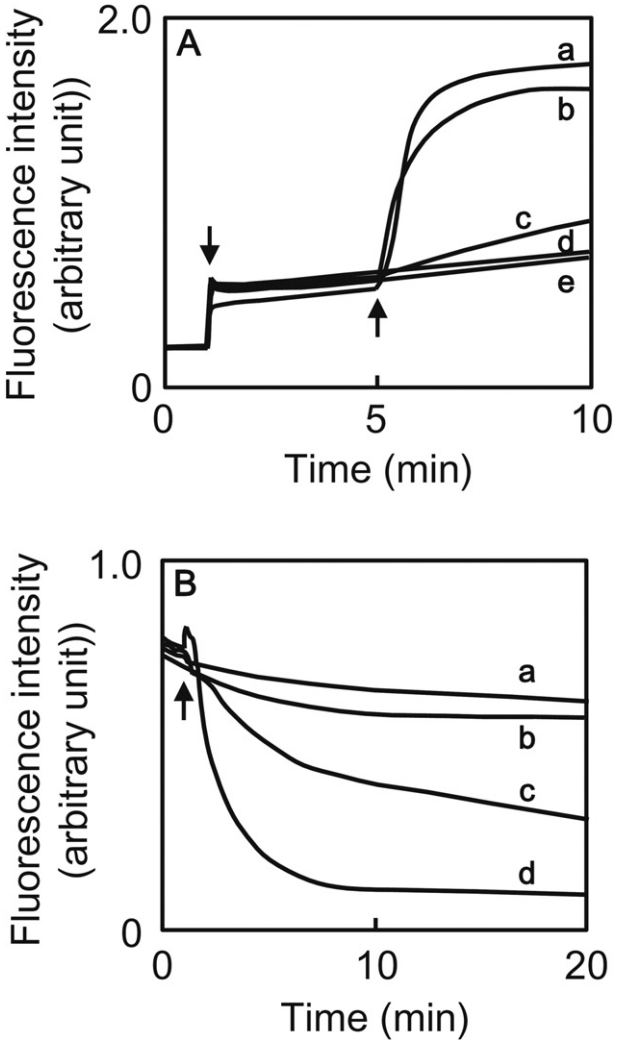

Fig. 4. Effects of the riccardin C derivatives on the inflow and outflow of ethidium on *S. aureus* N315. (A) Ethidium inflow. a, 5 μg/ml RC-112; b, 0.01% Triton-X; c, 50 μM CCCP; d, 40 μg/ml IDPO-9; e, 0.25% DMSO. EtBr (5 μg/ml) was added at 1 min (downward arrow) and compounds were added at 5 min (upward arrow). (B) Ethidium outflow. a, 0.25% DMSO; b, 40 μg/ml IDPO-9; c, 100 μg/ml SDS; d and 5 μg/ml RC-112. Compounds were added at 1 min (upward arrow).

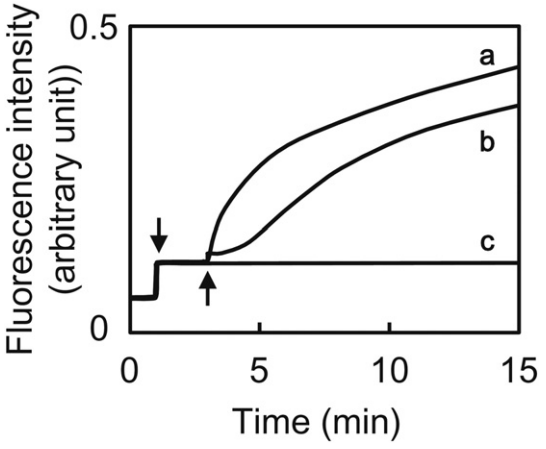

Fig. 5. Effects of the riccardin C derivatives on the fluorescence intensity of propidium iodide incubated with *S. aureus* N315: a, 100 μg/ml SDS; b, 15 μg/ml RC-112; c, 0.25% DMSO. Propidium iodide (10 μg/ml) was added at 1 min and compounds were added at 3 min.

seemed that the *B. subtilis* membrane had some defects by riccardin C derivatives. Although no significant morphological changes were observed in *S. aureus* (Fig. 6), TEM revealed the presence of intracellular lamellar mesosomal-like structures in RC-112-treated cells (Fig. 7). Membrane defect was previously shown to induce the development of mesosomes in cells treated with essential oil, gramicidin S, and defensin [29–31]; therefore, these findings supported the ability of RC-112 to affect membranes in *B. subtilis* and *S. aureus*.

3.7. Measurement of intracellular Na^+ and K^+ concentrations

Based on the TEM images, the membrane was thought to be affected by RC-112. As we could not find any visible changes in the membrane, this defect may not have been structural; however, its function as a barrier for small molecules was adversely affected. Figs. 4 and 5 showed that the permeability of the membrane to ethidium and propidium was increased. Therefore, the intracellular concentrations of smaller molecules, such as Na^+ and K^+, were measured (Fig. 8). In living cells, intracellular Na^+ concentrations are maintained at lower levels than extracellular concentrations, while K^+ concentrations are higher. When cells were treated with RC-112, intracellular Na^+ concentrations were increased and K^+ concentrations were decreased. A similar phenomenon was observed when cells were treated with valinomycin (K^+ ionophore), CCCP (H^+ ionophore). This result revealed that although the membrane defect induced by RC-112 was not structural, the function of the barrier was impaired.

4. Discussion

We performed several analyses to clarify the action mechanisms of riccardin C and its derivatives. Survival assay revealed that RC-112, AA-99, and AA-100 exhibited bactericidal effects, while IDPO-9 had bacteriostatic effects. IDPO-9 did not cause the inflow or outflow of ethidium, whereas the other derivatives did. IDPO-9-resistant mutants did not show cross-resistance to RC-112, AA-99, or AA-100. These results indicated that the action mechanism of RC-112 (also AA-99 and AA-100) differed from that of its partial structure, IDPO-9.

The chemical structure of IDPO-9 is similar to triclosan, a well-known antiseptic reagent. A previous study reported that triclosan inhibited FabI, an enoyl-acyl carrier protein reductase, and identified the action site of triclosan on FabI [22]. When IDPO-9 was displayed in the molecular model of FabI already reported [22], IDPO-9 could be placed in the catalytic pocket, similar to triclosan (Fig. 3), which implied that the action site of IDPO-9 was identical or very close to that of triclosan. This indication was also supported by the cross-resistance between IDPO-9 and triclosan on their resistant mutants. When we predicted the mutated FabI (A95V and F204S) based on the crystal structure of the wild type FabI, both IDPO-9 and triclosan were predicted to not fit securely (Fig. 3). As such, we believe that the target of IDPO-9 is FabI identical to that of triclosan. On the other hand, the tertiary structure of RC-112 may be bulkier than that of triclosan and IDPO-9. Neither triclosan-resistant mutants nor IDPO-9-resistant mutants showed resistance to RC-112, which indicated that the target of RC-112 may not be FabI.

RC-112 caused the inflow and outflow of ethidium. Since the active efflux of ethidium is generally mediated via multidrug efflux pumps [32], the depletion of a driving force, such as an H^+-motive force, may cause the inflow of ethidium. Of the multidrug efflux pumps identified in *S. aureus*, NorA, NorB, MdeA, MepA, SdrM, and LmrS have been shown to induce the efflux of ethidium [14,33–37]. Since all these pumps utilize an H^+-electrochemical gradient for transport, an increase in H^+ permeability in the cell membrane may lead to the disappearance of the H^+-electrochemical gradient, causing a loss of their function, and the 'apparent inflow' of ethidium. This inflow may also be caused by the inhibition of efflux pumps themselves. However, the apparent outflow

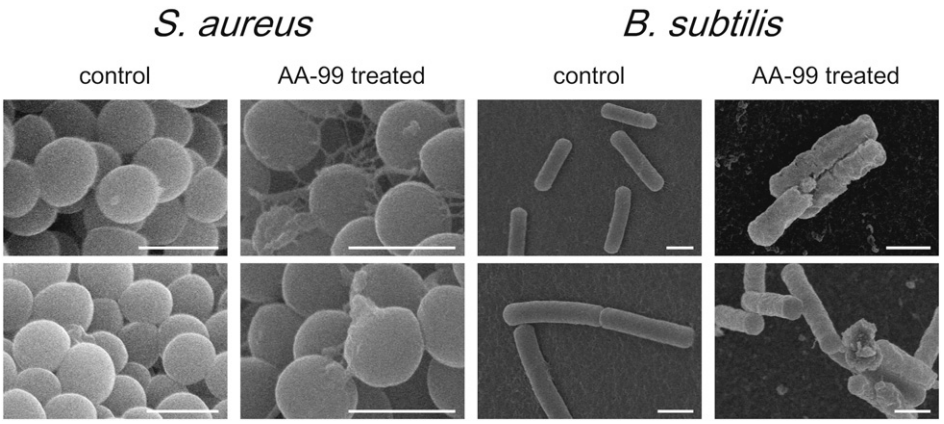

Fig. 6. Scanning electron microscopy images of untreated and treated *S. aureus* N315 and *B. subtilis* 168 cells. *S. aureus* and *B. subtilis* cells incubated in the presence of 0.8% DMSO (control) or 8 μg/ml AA-99 (AA-99 treated) for 3 h. Scale bar, 1 μm.

of ethidium may not occur even if the efflux pumps are inhibited. Thus, both the inflow and outflow of ethidium suggest that RC-112 may not be an inhibitor of efflux pumps. This proposal is supported by similar findings that were obtained when propidium iodide, which may not be a substrate for efflux pumps, was used.

Some morphological changes were observed on RC-112 treated cells. TEM revealed a thinner cell membrane and lower electron density on the membrane. The formation of mesosomes was also observed in many RC-112 treated cells. Mesosomes are thought to be intracellular structures that are formed by invaginations in the cell membrane [38], and are known to be caused by membrane damage, DNA damage, and the inhibition of protein synthesis. The appearance of mesosomes indicates that some membrane defects have occurred. Although the process of mesosome formation remains to be clarified, some studies have suggested the involvement of antimicrobial agents in the relationship between mesosome formation and membrane damage [29,39,40]. Based on these findings, the action mechanism of the riccardin C derivatives may be to induce cell leakage.

Several ionophores and antibacterial peptides are known to cause membrane defect. One of the carrier ionophores, valinomycin is a cyclopeptide antibiotic, and it has twelve carbonyl groups essential for binding of metal ions. Monensin is a polyether antibiotic, and it forms a complex with monovalent cations. Riccardin C derivatives (RC-112, AA-99, AA-100) actually have only one ether linkage (Fig. 1), so it might not bind to monovalent cations, and not be a carrier ionophore. Zervamicin IIB is a peptide antibiotic, which forms ion channels [41]. Its monomeric molecules bind to the membrane, inserted into lipid bilayers in the presence of membrane potential, and aggregate. From its chemical structure, riccardin C derivatives would be different from these ionophores. We previously showed the obvious structure–activity relationship of riccardin C derivatives [6,7]. This fact reminds us that riccardin C derivatives may bind to a peculiar target molecule on the

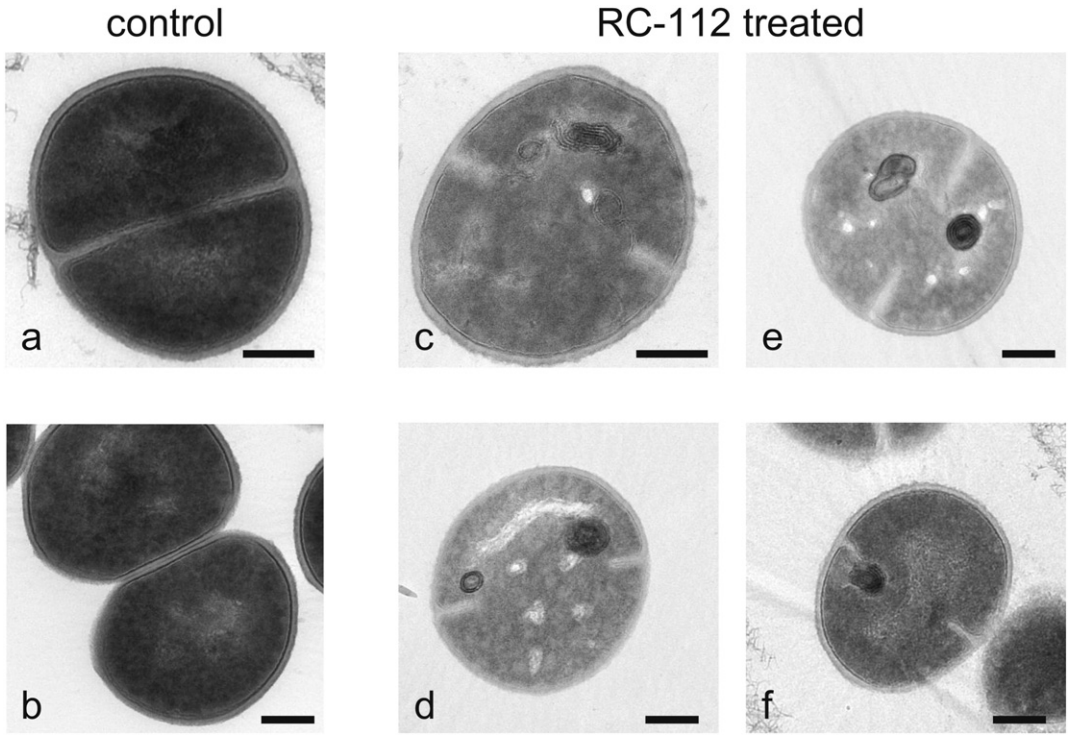

Fig. 7. Transmission electron microscopy images of untreated and treated *S. aureus* N315 cells. (a, b) *S. aureus* cells incubated in the presence of 0.8% DMSO (control); (c, d, e, f) *S. aureus* cells incubated in the presence of 8 μg/ml RC-112. Scale bar, 0.2 μm.

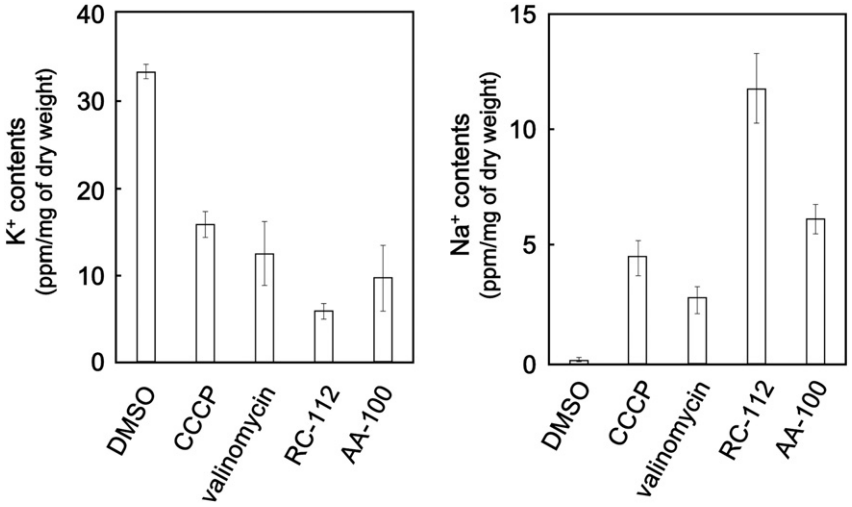

Fig. 8. Effect of RC-112 on intracellular Na^+ and K^+ concentrations. DMSO, 0.5%; CCCP, 10 μg/ml; valinomycin, 10 μg/ml; RC-112, 10 μg/ml; AA-100, 8 μg/ml. After a 90-min incubation with various agents, K^+ and Na^+ concentrations were measured.

membrane, not at random. Moreover, the target molecule might not be abundant on the membrane of *S. aureus* since significant changes were not observed (Fig. 6). Now, we have been trying to identify its target molecules.

Resistance to membrane defect appears to require a higher expression of efflux pumps or a change in the constitution of the membrane. However, this seems to be rare because it's not always true that the compounds are the substrates of the efflux pumps. Furthermore, changing the constitution of the membrane may not be advantageous for the survival of bacteria. We did not succeed in isolating RC-112-resistant mutants. These results indicate that riccardin C derivatives may be the seeds for potential antibacterial agents.

Other antimicrobial peptides have been shown to be able to impair the cell membrane and also possess other abilities [42]. Therefore, RC-112 derivatives may have dual targets for their antimicrobial activities. We recently reported the chemical synthesis of novel RC-112 derivatives and their anti-MRSA activities [6,7]. Further analyses of these derivatives are required to elucidate their structure–activity relationships and mechanisms of membrane damage in more detail.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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