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In Vitro Antimicrobial Activity of Aminoreductone against the Pathogenic Bacteria Methicillin-Resistant *Staphylococcus aureus* (MRSA)

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ABSTRACT: In this study, antimicrobial activity of aminoreductone (AR), a product formed during the initial stage of the Maillard reaction, against methicillin-resistant *Staphylococcus aureus* (MRSA) was evaluated. The significant growth inhibition of all 51 MRSA isolates irrespective of drug susceptibility by AR was observed. The minimum inhibitory concentration (MIC) of AR ranged from 13 to 26 mM. The bactericidal activity of AR was evaluated by a killing assay with multiples of MIC, and it was recognized to depend on its dose. The combined effects of AR and antibiotics frequently used for the treatment of infections caused by Gram-positive and Gram-negative bacteria, such as amikacin (AN), ciprofloxacin, imipenem and levofloxacin, were examined. As a result, AR did not interfere with these antibiotic activities against 12 MRSA isolates selected and showed the advanced effect of growth inhibition in combination with antibiotics. Moreover, the inhibitory effects of AR were similar to those of AN, an antibiotic with known adverse effects, some serious. These findings show that AR is a naturally formed antimicrobial agent present in thermally processed foods with potential health benefits in medical practice.

KEYWORDS: methicillin-resistant *Staphylococcus aureus*, MRSA, aminoreductone, Maillard reaction, antimicrobial activity, MIC

1. INTRODUCTION

Staphylococcus aureus (*S. aureus*), the most significant human pathogen,[1] colonizes in the nares and skin of about a third of all healthy people.[2] Under certain conditions (such as injury, transplant, chronic underlying disease, or immunocompromised conditions), *S. aureus* can cause a variety of serious infections, including bacteremia, endocarditis, sepsis, hospital-acquired pneumonia, vertebral osteomyelitis, abscess and surgical wound infections.[1] Although most staphylococcal infections can be successfully treated with antibiotics, reports of strains resistant to most available treatments are of great concern.[3] In the last three decades, strains of methicillin-resistant *S. aureus* (MRSA) isolated soon after antibiotic treatment have become a persistent problem worldwide.[4] Microbial resistance to antibiotics emerged soon after their first use in clinical practice and continues to pose a significant challenge for the health care sector.[5] Thus, the identification of new antimicrobial agents is a top research and development priority among scientists and pharmaceutical companies.[6]

The Maillard reaction, a heat-treatment-induced chemical reaction between amino and carbonyl groups, is significant for foods because it strongly affects the quality and acceptance.[7] The Maillard reaction initiates with the condensation of amino and carbonyl groups and leads to the final polymerized products through the formation of numerous intermediate products.[7,8] Several studies have reported the beneficial effects of advanced Maillard reaction products (MRP) such as melanoidins, including antioxidative,[9] antimicrobial,[10−13] antihypertensive,[9,14] antimutagenic, and anticarcinogenic properties.[15] As for the antimicrobial activity, several reports highlighted the *in vivo* and *in vitro* roles of melanoidins against *Bacillus stearothermophilus*, a highly thermoresistant, food-degradative microorganism,[11,16] and also against some pathogenic, spoilage-causing bacteria frequently found in food such as *Escherichia coli*,[9,10,12,13,17] *S. aureus*,[10,12,17] *Salmonella enteritidis*,[10] and *Bacillus subtilis*.[17]

The formation of aminoreductone (AR) (1-[N^{ε}-(N^{α}-acetyllysinyl)]-1,2-dehydro-4-deoxy-3-hexulose) in the initial stage of the Maillard reaction was first reported by Pischetsrieder et al.[18] in a heating solution of lactose and N^{α}-acetyllysine. Because AR can be detected after only a short period of heating, it is an important indicator for estimating the extent of the Maillard reaction and heat treatment of food.[18,19] Elucidation of the role and characteristics of AR are, therefore, of great interest to food scientists. So far, an antioxidant activity,[20] a protective effect on photodegradation of riboflavin,[21] and an antimicrobial activity against *Helicobacter pylori*, a Gram-negative bacillus[22] of AR (1-(butylamino)-1,2-dehydro-1,4-dideoxy-3-hexulose) derived from the Maillard reaction of lactose and butylamine were reported. Thus, we hypothesized that AR interferes with certain physiological behaviors such as viability and growth of MRSA, a Gram-positive coccus. In an attempt to seek alternative agents to antibiotics, and to further explicate the functional properties of

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Table 1. Inhibitory Effects of Aminoreductone on MRSA

		inhibition zone[a] (mm)						
no.	strain	AR (2.5 mg disk^{-1})	AR (2.5 mg disk^{-1}) and Cu^{2+} (5 μg disk^{-1})	LVX (5 μg disk^{-1})	CIP (5 μg disk^{-1})	IPM (10 μg disk^{-1})	AN (30 μg disk^{-1})	MIC (mM)
				(D)				
1	MRSA 105	20.5	7	nd[b]	nd	nd	18	20
2	MRSA 109	20	nd	nd	nd	nd	19	22
3	MRSA 115	16.5	nd	nd	nd	nd	17	16
4	MRSA 116	18	nd	nd	nd	nd	18	20
5	MRSA 117	19	nd	nd	nd	nd	16	14
6	MRSA 120	16	nd	nd	nd	nd	15	19
7	MRSA 121	15	7	nd	nd	nd	15	20
8	MRSA 122	15.5	7	nd	nd	nd	16	24
9	MRSA 123	16.5	7	nd	nd	nd	16	22
10	MRSA 124	16.5	7	nd	nd	nd	17	14
11	MRSA 125	15.5	8	nd	nd	nd	15	14
12	MRSA 126	16.5	9	nd	nd	nd	15	18
13	MRSA 127	18.5	9	nd	nd	nd	18	18
14	MRSA 136	18	9	nd	nd	nd	19	14
15	MRSA 141	20	7	nd	nd	nd	21	24
16	MRSA 143	18	8	nd	nd	nd	20	26
17	MRSA 145	20	8	nd	nd	nd	20	26
18	MRSA 148	19	9	nd	nd	nd	19	26
average								19.83 ab[c]
				(B)				
19	MRSA 100	17.5	nd	nd	nd	14	12	22
20	MRSA 102	20.5	nd	nd	nd	54	21	24
21	MRSA 103	21.5	7	15	nd	nd	16	22
22	MRSA 128	18.5	8	8	nd	nd	17	18
23	MRSA 129	18.5	9	nd	nd	24	20	20
24	MRSA 131	18	7	12	nd	nd	17	24
25	MRSA 132	20.5	8	16	nd	nd	18	24
26	MRSA 133	20.5	9	12	nd	nd	18	14
27	MRSA 140	20	8	19	nd	nd	21	22
28	MRSA 142	20	9	nd	nd	60	24	22
29	MRSA 144	20	10	nd	nd	32	26	18
30	MRSA 149	16	8	nd	nd	32	18	26
average								21.33 a
				(C)				
31	MRSA 1	23.3	nd	16	13	nd	19	13
32	MRSA 101	19.5	nd	14	nd	30	17	20
33	MRSA 106	18	7	18	13	nd	17	14
34	MRSA 108	18.5	nd	22	nd	24	22	14
35	MRSA 130	20	8	14	nd	9	17	18
36	MRSA 134	18	8	8	nd	30	18	18
37	MRSA 139	19	9	16	nd	8	19	20
average								16.71 b
				(D)				
38	MRSA 107	16.5	8	28	30	48	21	18
39	MRSA 110	17	7	28	24	34	22	20
40	MRSA 111	18.3	nd	31	23	30	15	17
41	MRSA 112	19.3	nd	31	32	22	15	18

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Table 1. Continued

		inhibition zone[a] (mm)						
no.	strain	AR (2.5 mg disk^{-1})	AR (2.5 mg disk^{-1}) and Cu^{2+} (5 μg disk^{-1})	LVX (5 μg disk^{-1})	CIP (5 μg disk^{-1})	IPM (10 μg disk^{-1})	AN (30 μg disk^{-1})	MIC (mM)
				(D)				
42	MRSA 113	16	7	20	15	30	17	16
43	MRSA 114	17.5	7	31	29	29	15	18
44	MRSA 118	18.5	7	30	31	27	16	18
45	MRSA 119	19	nd	32	29	28	22	18
46	MRSA 135	17.5	9	31	31	16	16	18
47	MRSA 137	19	8	20	8	36	20	18
48	MRSA 138	20	9	13	8	10	19	24
49	MRSA 146	20	8	32	30	18	22	20
50	MRSA 147	18	8	31	32	22	22	26
51	MRSA 104	20.5	nd	18	13	8	16	24
average								19.50 ab

[a] Diameter of each disk was 6 mm, and values are mean of duplicate LVX, levofloxacin; CIP, ciprofloxacin; IPM, imipenem; AN, amikacin. [b] Not determined. [c] Values in averages of groups are significantly different ($P < 0.05$) unless followed by the same letters.

AR, this study focused on investigating the effects of AR against the pathogenic microorganism MRSA.

2. MATERIALS AND METHODS

2.1. Reagents. Mueller−Hinton broth (MHB) was obtained from Becton, Dickinson and Company (Cockeysville, MD). Commercially available standard disks (φ = 6 mm) of amikacin (AN: 30 μg disk^{-1}), ciprofloxacin (CIP: 5 μg disk^{-1}), imipenem (IPM: 10 μg disk^{-1}), and levofloxacin (LVX: 5 μg disk^{-1}) were also obtained from Becton, Dickinson and Company. Lactose monohydrate was purchased from Nacalai tesque, Inc. (Kyoto, Japan). *n*-Butylamine and agar were obtained from Wako Pure Chemical Industries (Osaka, Japan). All other reagents were of the highest grade commercially available. Milli-Q water or sterilized water was used in all procedures.

2.2. Preparation of AR and Its Degradation Product. Purified AR was prepared according to our previous reports.[20,23] Briefly, lactose monohydrate (262 mM) and butylamine (1.16 M) were dissolved in 1.28 M phosphate buffer (pH 7.0). The sample solution (10 mL) was heated at 100 °C for 15 min, and immediately cooled on ice. The heated sample solution was extracted three times with a double volume of ethyl acetate, and the ethyl acetate layer was evaporated to dryness under reduced pressure. The residue was dissolved in 10 mL of 20% methanol and filtered through a Sep-Pak Plus C18 cartridge (Waters Corporation, Milford, MA) (activated by 5 mL of ethanol and equilibrated using Milli-Q water) to remove brown components (melanoidin). The clear eluate was evaporated again and freeze-dried under reduced pressure to collect the purified AR. In a previous study, Shimamura et al.[23] reported the ^{13}C and ^{1}H NMR data on this extracted product, and those signals could be assigned to the AR (1-(butylamino)-1,2-dehydro-1,4-dideoxy-3-hexulose). The concentration of AR was calculated with the extinction coefficient of AR (17.98 M^{-1} cm^{-1}) at 320 nm.[21]

In the final stages of the Maillard reaction, the brown, high-molecular-weight polymerized products are formed. It is so-called melanoidins by the French chemist Louis Camille Maillard.[7] Because melanoidins possessed antimicrobial activity against several pathogenic strains, as described above,[9−13,16,17] the AR-derived MRP which was a mixture of advanced MRP mainly including melanoidins was also used in this study. AR-derived MRP was prepared as follows: the purified AR solution was kept at room temperature for at least 3 weeks until the remaining amount of AR was less than 0.5% confirmed by the XTT assay or by estimating the absorbance at 320 nm.[22]

2.3. Bacterial Strains and Culture Conditions. Fifty-one randomly chosen MRSA isolates obtained from patients in Kochi Medical School Hospital (Kochi, Japan) were used in this study. MRSA isolates were grown on the MHB agar plates supplemented with 1.4% agar and incubated at 37 °C under aerobic conditions for 24 h. Whenever appropriate, MHB liquid medium was used in this study.

2.4. Disk Diffusion Susceptibility Methods. The growth inhibition of MRSA by AR using the filter paper disk diffusion method on the MHB agar plate incubated at 37 °C under aerobic conditions was assessed.[24] Purified AR was diluted in Milli-Q water and dropped to the disk. Sterilized standard disks (φ = 6 mm) containing 2.5 mg of AR were placed on the MHB agar plates previously spread with 0.1 mL of bacterial suspension (OD_{600} = 0.1) in MHB liquid medium. The plates were incubated for 24 h at 37 °C under aerobic conditions. The inhibition zones were measured and recorded in millimeters, and the average diameter of at least two repetitions was calculated. The entire diameter of inhibition zone, including the diameter of the disk, was measured. Four standard commercially available antibiotic disks, namely, AN (30 μg disk^{-1}), CIP (5 μg disk^{-1}), IPM (10 μg disk^{-1}), and LVX (5 μg disk^{-1}), were also utilized in this assay.

Previously, Shimamura et al.[19] observed that the addition of Cu^{2+}, a strong oxidizing agent, could drastically decrease the AR content in the lactose−butylamine model solution. Therefore, to confirm the contribution of AR to the inhibitory activities against MRSA, Cu^{2+} (5 μg) was added into the disk containing AR (2.5 mg) and the changes of the inhibition zone were observed in this study.

2.5. Microscopical Observation. The MRSA isolates grown at the peripheral area in the inhibition zones with 4 antibiotics and AR by disk diffusion assay were subjected to microscopical examination. Briefly, aliquots of the isolates exposed to 4 antibiotics and AR were fixed on the slide glass with ethanol, stained with neo-B&M Gram staining kit (Wako Co., Ltd., Osaka, Japan) and observed with an objective lens (×100) for morphological characteristics.

2.6. Determination of Minimum Inhibitory Concentrations (MIC). MIC of AR against MRSA was determined using an agar

dilution method described previously.[24,25] Purified AR was diluted in Milli-Q water. 750 μL of AR solution at given concentrations was separately added to each dish containing 14.25 mL of yet-not-solidified MHB agar. The final concentrations of AR in the agar plates ranged from 0 to 30 mM. Subsequently, each 10 μL of MRSA suspension (OD_{600} = 0.1) was serially 10-fold diluted and inoculated onto the surface of the AR-supplemented agar plates and then incubated at 37 °C for 48 h under aerobic conditions. Sterilized water was used as a control for all experiments. The number of colony forming units (CFU) was determined as a measure of bacterial viability.[26] MIC was defined as the lowest AR concentration to inhibit 1×10^4 CFU compared to that of controls. In addition, the AR-derived MRP was also used and compared with AR in this study. All tests were performed in duplicate at least.

2.7. Killing Assay. To determine the bactericidal activity of AR against MRSA, killing experiments were performed in the presence of 5, 10, or 15 × MIC of AR, according to the methods described previously.[25] Bacteria, well grown after being cultured for 24 h in MHB liquid medium and corresponding to the late exponential phase, were harvested, washed with MHB liquid medium and centrifuged at 8000*g* for 1 min (KUBOTA 1120, Kubota Corp., Tokyo, Japan) to remove the supernatant. In 1.5 mL centrifuge tubes, 0.4 mL of the bacterial suspension (10^8 cfu mL^{-1}) in fresh MHB liquid medium with or without AR (control) was incubated under aerobic conditions with shaking (Bio shaker BR-40LF, Taitec Co., Ltd., Saitama, Japan) at 37 °C for 6 h. At 2, 4, and 6 h after incubation, each 10 μL of the suspension was serially 10-fold diluted and inoculated onto the MHB agar plates and cultured for 24 h under aerobic conditions to determine the viability. The ability of AR to kill MRSA strains was evaluated by CFU counts and comparison with controls. All examinations were performed in duplicate at least.

2.8. Cluster Analysis. For all strains, cluster analysis of the inhibitory effects of AR and antibiotics was performed based on the measurement of inhibition zones by a tree joining algorithm (complete linkage and squared Euclidean distance)[27] using STATISTICA software (StatSoft, Inc. Tulsa, OK).

3. RESULTS AND DISCUSSION

3.1. Inhibitory Effects of AR against MRSA. The inhibitory effects of AR against 51 MRSA isolates were examined by the standard disk diffusion and agar dilution methods, which are widely used to study the bioactivity of chemical compounds. The inhibition zone for each isolate by AR (2.5 mg) is shown in Table 1A−D. Considering the drug-susceptibility test using the LVX, CIP, and IPM, MRSA isolates were classified into four groups (A−D): group A comprised 18 isolates resistant to all 3 antibiotics (Table 1A), group B comprised 12 isolates resistant to 2 of 3 antibiotics (Table 1B), group C comprised 7 isolates resistant to 1 of 3 antibiotics (Table 1C), and group D comprised 14 susceptible isolates (Table 1D). Inhibition zones ranged from 15 mm (MRSA 121) to 23.3 mm (MRSA 1) in diameter, suggesting that all isolates exhibited sensitivity to AR.

Previously, it was reported that the addition of Cu^{2+} could drastically reduce the concentration of AR because a labile reductone structure in AR could be readily oxidized.[19] Hence, to clarify whether AR was responsible for the antimicrobial ability against MRSA or not, 5 μg of Cu^{2+} was added to the disk containing 2.5 mg of AR and the effect was evaluated. In the presence of Cu^{2+}, considerable decrease of the inhibition zones was recognized for all isolates (Table 1A−D) because of decreased AR concentration. On the other hand, the disk containing 5 μg of Cu^{2+} alone did not exhibit an inhibitory effect against MRSA, indicating that AR *per se* possessed the potential to inhibit the growth of MRSA.

As described above, AR has the labile reductone structure and can easily change to intermediate and advanced MRP. Finally, the melanoidins, which were reported to be antimicrobial compounds, were formed. Melanoidins, high-molecular-weight compounds, are present in widely consumed dietary food and exhibit antimicrobial activity *in vitro*.[9−13,16,17] Thus, AR-derived MRP including melanoidins was also tested in the growth inhibition assay at a concentration similar to each MIC value of AR as described below. However, contrary to our expectation, no inhibitory effect was observed (data not shown). Thus, we concluded that the AR-derived MRP containing melanoidins showed no effect against MRSA at tested concentration.

Furthermore, the inhibitory effects of AR against 51 MRSA isolates were confirmed by an agar dilution method, demonstrating that all isolates exhibited susceptibility to AR at concentrations lower than 26 mM (Table 1A−D). The MIC values ranging from 13 mM (MRSA 1) to 26 mM (MRSA 143, 145, 147, 148 and 149) were proven in all 51 isolates tested.

Fluoroquinolone antibacterial agents, such as LVX[28] and CIP,[28] β-lactam antibiotics, such as IPM,[29] or aminoglycoside antibiotics, such as AN,[30] are widely used alone or in combination with other antimicrobial agents for the treatment of several serious infections caused by Gram-negative and Gram-positive bacteria. The susceptibility of MRSA to these four antibiotics compared with AR was investigated by using standard commercial disks (Table 1A−D). Interestingly, all isolates were susceptible to AR (2.5 mg) as well as AN (30 μg). In groups B and C, no isolate representing CIP-sensitive and LVX-resistant was observed, indicating that among fluoroquinolone antibiotics, LVX was a more effective growth inhibitor of MRSA than CIP. Analysis of variance (ANOVA)[27] was used to test the differences at the MIC mean values of AR among the 4 groups. There was a statistically significant difference between groups B and C (α = 0.05, P = 0.046). However, similarity was found first among groups A, C and D (α = 0.05, P = 0.181) and then among groups A, B, and D (α = 0.05, P = 0.623). Based on these results, it could be concluded that no correlation between the MIC values and groups existed.

As a result, the inhibitory effect of AR affected all isolates irrespective of drug-susceptibility properties. This result suggested that AR could be used both as an alternative agent and as an adjuvant therapy for MRSA infection. MRSA, widely resistant to antibiotics, has adapted to survive treatment with many kinds of antibiotics and is especially troublesome in nosocomial infection because of difficulty and failure to eradicate the infection with antibiotics. This forces us to seek alternative remedies. The effective antimicrobial activity of AR, a natural product formed during food processing, may provide insight to scientists searching for these alternative remedies.

3.2. Killing Ability of AR against MRSA. As described above, growth inhibition of MRSA by AR was recognized for all isolates tested. The *in vitro* activities of AR were evaluated further by determining the bactericidal activity on 11 isolates chosen randomly from 4 groups, including MRSA 1 (lowest MIC: 13 mM), 143 (highest MIC: 26 mM) and other 9 isolates with varying drug susceptibilities. The killing assay was performed in the presence of 5, 10, or 15 × MIC of AR. The killing assay has been proposed as the most reliable method for determining the susceptibility of microorganisms to compounds and antibiotics.[25]

Table 2. Killing Ability of Aminoreductone against MRSA Strains[a]

strain/AR concn	susceptibility	5 × MIC	10 × MIC	15 × MIC	bactericidal concn (mM)
MRSA 1	IPM	−	+	+	130
MRSA 100	LVX, CIP	−	+	+	220
MRSA 102	LVX, CIP	−	−	+	360
MRSA 106	IPM	−	−	−	390
MRSA 113	susceptibility	−	+	+	160
MRSA 117	LVX, CIP, IPM	−	+	+	140
MRSA 128	CIP, IPM	−	−	+	270
MRSA 131	CIP, IPM	−	+	+	120
MRSA 132	CIP, IPM	−	−	+	360
MRSA 138	susceptibility	−	+	+	240
MRSA 143	LVX, CIP, IPM	−	−	+	390

[a] Bactericidal (+) and no bactericidal (−) effects observed at 6 h after exposure is shown. LVX, levofloxacin; CIP, ciprofloxacin; IPM, imipenem.

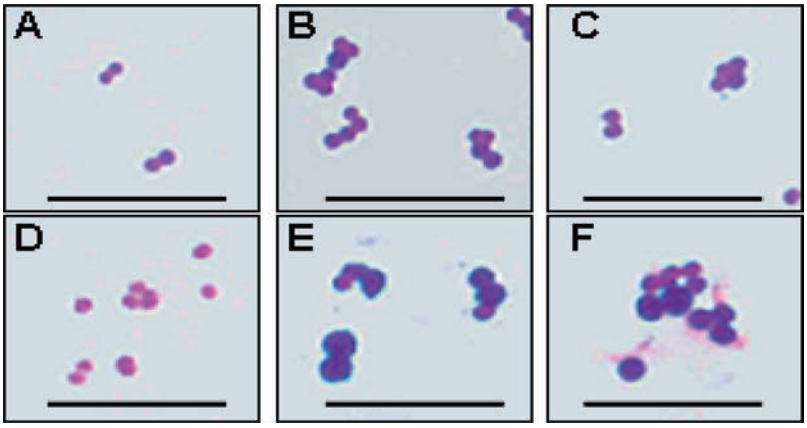

Figure 1. Microscopical observations of MRSA exposed to AR and antibiotics.
MRSA is a Gram-positive (blue to purple color) small coccus (A). There are no different aspects of MRSA between exposure to AR (B) and AN (C) compared to untreated isolate (A). The isolates exposed to IPM changed to Gram-negative (red color) cocci (D). Exposed with fluoroquinolones (LVX (E) and CIP (F)), the Gram-positive small cocci converted to large cocci. Bar is 10 μm.

The bactericidal effects against MRSA after exposure to AR were examined (Table 2). The bactericidal effects on all 11 isolates were observed at a concentration of less than 390 mM. The most potent bactericidal activity was exhibited for MRSA 1 at a concentration of 10 × MIC (130 mM) of AR. For all isolates, the bactericidal activity of AR was attained at a much higher concentration than MIC concentration such as 10 or 15 × MIC of AR. Almost all strains were killed at an AR concentration of 15 × MIC, whereas only MRSA 106 isolate required over 15 × MIC. These results indicated that the killing effect of AR differs among individual clinical isolates and is not associated with the drug-susceptibility properties of MRSA. Furthermore, these results also suggested that MIC of AR achieved antimicrobial activity through bacteriostatic effect, while higher doses were required for bactericidal activity. This indicated that the bactericidal effect of AR against MRSA was dose-dependent, similar to antibiotics such as AN, LVX and CIP.

The exact mechanism by which MRP affects bacterial growth is not yet known. However, it has been suggested that high-molecular-weight compounds like melanoidins with anionic charges could develop their antimicrobial activity by binding essential metals, such as iron, copper, and zinc, which are key elements in metabolism and essential for the growth and survival of pathogenic bacteria.[12,13,17] In addition, other studies attributed the antimicrobial activity of the Maillard reaction compounds to their interference with the uptake of serine, glucose, and oxygen, thereby inhibiting the sugar catabolizing enzymes of microorganisms by causing irreversible changes in both the inner and outer cell membranes followed by the inhibition of nutrient transport.[16] Rufián-Henares and Morales[13] reported that the growth inhibitory activity and bactericidal activity against *Escherichia coli* increased depending on the molecular weight of MRP (<3 kDa, 3−10 kDa, and >10 kDa). Einarsson et al.[10] also showed that the growth inhibitory activity of MRP with more than 1 kDa molecular weight was higher than that with less than 1 kDa molecular weight. As described above, so far, most attention was paid for high-molecular-weight MRP such as melanoidins on the topic of antimicrobial activity. Thus, it is particularly noteworthy that AR possesses bactericidal ability against MRSA. Our findings provide a novel perception that low-molecular-weight MRP such as AR (217 Da) can contribute to exhibit bacteriostatic and bactericidal activity against MRSA. AR from lactose and butylamine showed a high reducing activity.[19] Lanciotti et al.[16] suggested that the inhibitory effect against *Bacillus stearothermophilus* seemed to be influenced by the reduction of the redox potential in the growth medium as a consequence of the addition of MRP with high

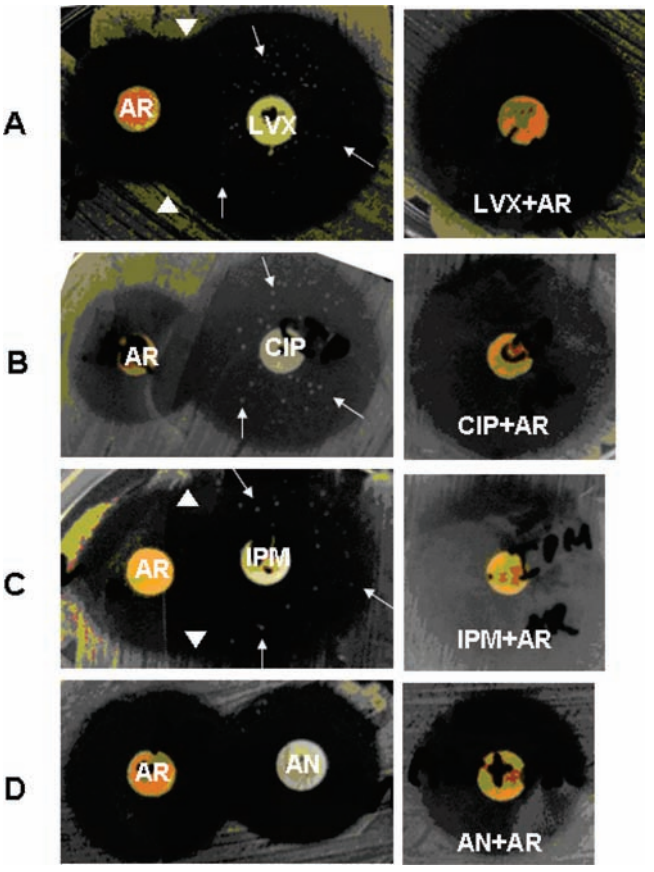

Figure 2. The inhibition zones of 4 antibiotic disks (LVX (A), CIP (B), IPM (C) or AN (D)) in combination with 2.5 mg of AR (left panel) and the mixtures of each antibiotic + AR (right panel) in MRSA isolate resistant to 3 antibiotics (LVX, CIP and IPM). Arrows denote colonies appearing within the inhibition zones induced by LVX (A), CIP (B) and IPM (C). Arrow heads (A and C) denote the obscure border areas due to fusion at contact point of the inhibition zones induced by AR and antibiotics (LVX and IPM), suggesting the synergistic effect to inhibit the growth. No colonies observed in the fused area in combinations (left panel) as well as the mixtures (right panel) indicates that AR has at least no antibiotic antagonism.

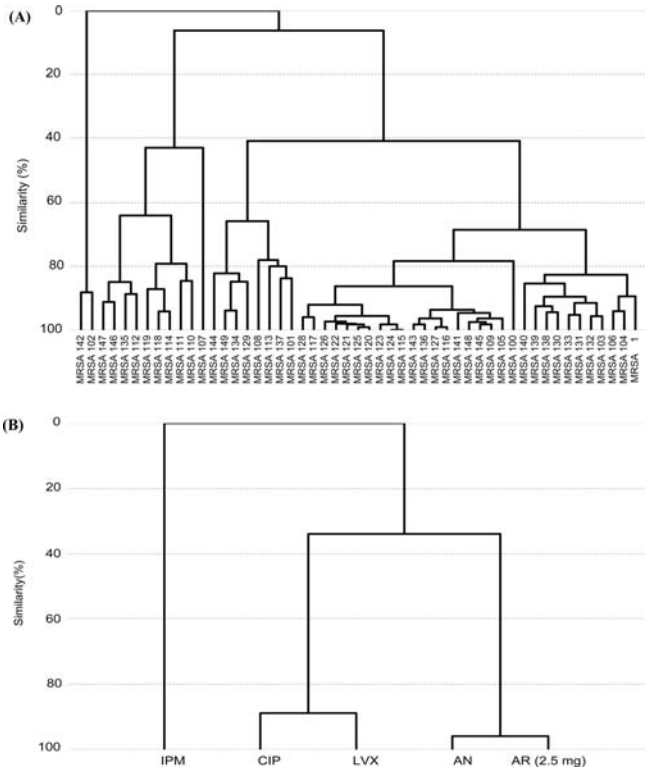

Figure 3. By disk diffusion assay, dendogram produced by cluster analysis of 51 MRSA isolates using the values of growth inhibition induced by AR (A) and 4 antibiotics (B). There was 95% similarity between AN (30 μg) and AR (2.5 mg).

reducing property. From this discussion, the effect of AR against MRSA might be partially caused by its reducing ability.

As the mechanism of action of AR against pathogenic bacteria has not yet been elucidated, we attempted to observe bacterial shape by Gram-staining of MRSA exposed to AR and antibiotics and compared their morphological characteristics (Figure 1). The isolates of MRSA, a Gram-positive small coccus, exposed to AR and AN were morphologically indistinguishable compared to untreated isolate (Figure 1A−C). However, Gram-positive was changed to Gram-negative by IPM (Figure 1D), suggesting that insufficient peptidoglycan synthesis resulted in the thin cell wall. The isolates exposed to LVX (Figure 1E) and CIP (Figure 1F) converted to large cocci due to probable fragility of cell wall and cell membrane unlike AR and AN. These results provided important clues in understanding the mechanisms by which AR functions against MRSA. These results provided important clues in understanding the mechanisms by which AR functions against MRSA. We report for the first time that AR exhibits growth inhibition and killing activity against MRSA here, raising the possibility that AR is a good candidate for preventing the threat caused by MRSA infection. Thus, food with the addition of synthesized AR could be used as a functional product for the prevention and treatment of MRSA infection.

3.3. Verification of the Antimicrobial Activity of AR in a Combination with Antibiotic. Combined antibiotic therapy has been shown to delay the emergence of bacterial resistance and may also produce desirable synergistic effects for treating of bacterial infections.[31] Drug synergism can, however, be beneficial (synergistic or additive interaction) or deleterious (antagonistic or toxic outcome).[31] The combined effect of AR and antibiotics were examined in 12 MRSA isolates selected as follows: MRSA 1 (lowest MIC: 13 mM), 143 (highest MIC: 26 mM), 100 (LVX and CIP resistant), 131 (CIP and IPM resistant), 117 (LVX, CIP, and IPM resistant), and other 7 MRSA isolates susceptible to antibiotics (107, 110, 113, 114, 119, 137, and 146). The disk diffusion assay utilizing individual antibiotic and AR disks and the disks containing the mixtures demonstrated no antibiotic antagonism (Figure 2), indicating that AR does not interfere with the activity of antibiotics used. In the case of drug-resistant MRSA, several colonies (resistant strains) appeared on the inhibition zone of the disks containing LVX, CIP, or IPM. However, no bacteria were observed on the inhibition zones of these antibiotic disks in combination with AR and the disks containing the mixtures (Figure 2), implying that the combination of antibiotics and AR is useful and may delay the emergence of bacterial resistance.

Despite the fact that the standard disk diffusion method is widely used to study the bioactivity of chemical compounds,[9] the ability of compounds to diffuse through the nutrient agar medium is a major limitation in the evaluation of the antimicrobial effects of those compounds. However, our results documented

that the border areas at contact point of inhibition zones between antibiotics (LVX and IPM) and AR were fused and obscure (Figure 2A,C), suggesting a synergistic effect to inhibit the growth. No synergistic effect was observed in combination between antibiotics (CIP and AN) and AR. These results lead us to consider combinatorial approaches based on foods containing AR to the prevention and treatment of MRSA infection.

3.4. Cluster Analysis. On the basis of the inhibition zones of AR and antibiotics, cluster analysis was performed, demonstrating that all isolates in group A were completely closed and isolates in group D were relatively closed, while isolates belonging to groups B and C dispersed (Figure 3A). Interestingly, these analyzed data deduce that AR probably exerts growth inhibition at a constant level to MRSA representing 3 drug-resistant and susceptible, but the growth inhibition level of AR was variable in other MRSA isolates. We also considered the similar or different effects of antimicrobial agents (AR and 4 antibiotics) in the growth inhibition of MRSA (Figure 3B). AR (2.5 mg) was present as 95% similar to AN (30 μg), whereas it was 72% different from CIP and LVX and 100% different from IPM in terms of growth inhibition. Differences in the effects of AR compared with 3 antibiotics (CIP, LVX, and IPM) suggested that the antibacterial functions of AR differ from the mechanisms caused by those antibiotics, such as disturbances in DNA fork progression and replication repair (LVX and CIP) or peptidoglycan synthesis inhibition (IPM).[29] Amikacin, an aminoglycoside, inhibits protein synthesis by "irreversibly" binding to the 30S ribosomal subunit to prevent the formation of an initiation complex with mRNA.[30] On the other hand, as for the antimicrobial function of AR, it is considered a possibility that AR possessing the reducing property leads to the reduction of the redox potential in the growth medium. However, the exact antimicrobial mechanism of AR is still unknown. The results from the killing assay suggested that AR and AN have each a different function as antimicrobial activity even in similar effect of growth inhibition. Of the 4 antibiotics used, AN showed the highest activity in the growth inhibition of MRSA but showed similarity in effect with AR, suggesting that AN could be used as a good reference during the investigation for the function of antimicrobial activity of AR. AN has been generally recognized to yield some serious adverse effects such as nephrotoxicity and permanent vestibular and/or auditory ototoxicity, hence the use of AR in place of AN could prove advantageous with reduced occurrence of serious adverse effects.

This is the first report to show that AR inhibits the growth and viability of MRSA, irrespective of drug susceptibility. In addition, AR showed bactericidal activity and the advanced effect in combinations with antibiotics. The antimicrobial effect of AR raises the possibility that foods containing AR are valuable sources of anti-MRSA compounds. Thus, investigating the mechanisms by which AR functions against MRSA is of significance in order to understand its extensive applications in health and medical fields. Foods containing AR, such as milk and dairy products, may be a promising and effective source for AR as an adjuvant. Overall, this study provides useful information for identifying the technological conditions favoring the formation of the Maillard reaction products, such as AR or melanoidin as functional ingredients in food.

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ABBREVIATIONS USED

S. aureus, *Staphylococcus aureus*; MRSA, methicillin-resistant *Staphylococcus aureus*; AR, aminoreductone; MIC, minimum inhibitory concentration; LVX, levofloxacin; CIP, ciprofloxacin; IPM, imipenem; AN, amikacin

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