Ambuic Acid Inhibits the Biosynthesis of Cyclic Peptide Quormones in Gram-Positive Bacteria

Jiro Nakayama,1* Yumi Uemura,1 Kenzo Nishiguchi,1 Norito Yoshimura,1 Yasuhiro Igarashi,2 and Kenji Sonomoto1,3

Department of Bioscience and Biotechnology, Faculty of Agriculture, Graduate School, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan; Biotechnology Research Center, Toyama Prefectural University, 5180 Kurokawa, Imizu, Toyama 939-0398, Japan; and Department of Functional Metabolic Design, Bio-Architecture Center, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan

Received 25 July 2008/Returned for modification 1 October 2008/Accepted 12 November 2008

Quorum sensing is a cell-density-dependent regulatory system in gram-positive bacteria and is often regulated by cyclic peptides called “quormones,” which function as extracellular communication signals. With an aim to discover an antipathogenic agent targeting quorum sensing in gram-positive bacteria, we screened 153 samples of fungal butanol extracts with the guidance of the inhibition of quorum-sensing-mediated gelatinase production in Enterococcus faecalis. Following the screenings, we found that ambuic acid, a known secondary fungal metabolite, inhibited the quorum-sensing-mediated gelatinase production without influencing the growth of E. faecalis. We further demonstrated that ambuic acid targeted the biosynthesis of a cyclic peptide quormone called gelatinase biosynthesis-activating pheromone (GBAP) (15). Although the molecular mechanism of GBAP biosynthesis has been suggested to be similar to that of the agr quormone, the organization of open reading frames involved in GBAP biosynthesis is somewhat different. It has been demonstrated that the GBAP propeptide is translated from fsrD, which is located in frame in the 5′-end reading frames involved in GBAP biosynthesis is somewhat different. It has been demonstrated that the GBAP propeptide is translated from fsrD, which is located in frame in the 5′-end

Quorum sensing is a common system present in unicellular microorganisms that controls the expression of certain target genes in a cell-density-dependent manner (38). In order to orchestrate this cell-to-cell communication, bacteria often use signal molecules, occasionally called “autoinducers,” “pheromones,” or “quormones” (we have called them “quormones” in this study). Many gram-positive bacteria employ peptides for quorum sensing, while gram-negative bacteria use nonpeptidic compounds such as N-acylhomoserine lactones in most cases (14, 38). Cyclic thiolactone and lactone peptides are commonly found quormones that are involved in quorum sensing in some gram-positive species (10, 33).

The agr system in staphylococci is the best-studied cyclic peptide-mediated quorum-sensing system (5, 20). This system employs 7- to 9-residue cyclic peptide quormones, called autoinducing peptide (AIP), in which the cysteine residue at the fifth position from the C terminus forms a thiolactone along with the C-terminal α-carboxyl group (5). A quormone propeptide is translated from agrD and is processed and cyclized with the aid of a biosynthetic enzyme, namely, AgrB (29). The mature quormone accumulated outside the cell triggers a two-component regulatory system through the activation of AgrC, which is a membrane histidine kinase (4). Eventually, AgrA, which is the response regulator, is phosphorylated and promotes the expression of RNA III, which encodes delta-hemolysin and also acts as a regulatory RNA molecule that controls the gene expression of staphylococcal virulence factors, e.g., hemolysins and toxic shock syndrome toxins (20). The four components, namely, AgrA, AgrB, AgrC, and AgrD, which are involved in agr quormone sensing, are encoded in a gene cluster organized as agrBDA (20).

An ortholog of an agr-like gene cluster was found in the genome sequences of some gram-positive bacteria including those belonging to the genera Listeria, Clostridium, and Bacillus, suggesting the widespread presence of cyclic-peptide-mediated quorum sensing among gram-positive bacteria (17, 29). For example, a “Lactobacillus agr-like gene module” (lam) was found in the genomic sequence of Lactobacillus plantarum WCFS1 (34). The structure of a putative cyclic peptide quormone was elucidated to be a 5-residue cyclic peptide in which the sulphydryl group of the N-terminal cysteine residue formed a thiolactone with the C-terminal α-carboxyl group (34). The putative cyclic peptide quormones LsrD698 and LsrD826 have been identified from Listeria innocua and are also suspected to function as quormones in Listeria monocytogenes because of the DNA sequence identity between these two species (19).

It is also known that Enterococcus faecalis possesses an fsr quorum-sensing system mediated by an 11-residue cyclic peptide carrying a lactone bridge between the hydroxyl group of serine residue at the third position and the C-terminal α-carboxyl group (15, 16, 27, 28). This cyclic peptide quormone secreted from each cell triggers the expression of two extracellular pathogenicity-related proteases, namely, gelatinase and serine protease, and was named gelatinase biosynthesis-activating pheromone (GBAP) (15).
part of fbr, and that FsrB', an FsrD segment-truncated FsrB, is functional as a cysteine protease-like processing enzyme to generate GBAP from FsrD (17).

Quorum sensing has recently been considered to be an emerging target for antimicrobial drug therapy (2, 6, 39). Compounds targeting quorum sensing can attenuate virulence without displaying any bactericidal or bacteriostatic activity and are proposed to be used as “antipathogenic drugs” (7, 22). For example, macrolides such as azithromycin, which inhibit N-acylhomoserine lactone-mediated quorum sensing but not the growth of *Pseudomonas aeruginosa*, are known to efficiently alleviate the symptoms of cystic fibrosis and diffuse panbronchiolitis (35–37). Furthermore, several other studies have already revealed the efficacy of inhibitors targeting the N-acylhomoserine lactone-mediated quorum sensing of gram-negative bacteria (7, 23, 30–32).

In the case of gram-positive pathogens, inhibitors of quorum sensing have been investigated mainly with regard to the staphylococcal *agr* system (6, 13, 20). Lyon et al. successfully developed a rational design of a peptide antagonist of the *agr* quorum (12, 13). A heptapeptide named RNA III-inhibiting peptide, which is found in the culture filtrates of some staphylococcal strains, and its synthetic analogs are also expected to be antistaphylococcal agents (1). Recently, 3-oxo-C12-homoserine lactone was reported to have successfully inhibited the *agr* quorum-sensing system (26). It has also been reported that the peptide inhibitor P+1, a proline-containing mimic peptide of the AgrD processing region, inhibited the removal of the N-terminal leader of the quorone peptide, which resulted in the blockage of the *agr* quorum-sensing system (8).

In order to discover compounds that inhibit *E. faecalis* quorum sensing, we have recently established a screening system for inhibitors targeting the GBAP-mediated quorum-sensing system, called the *fsr* system, and found that a peptide antibiotic, namely, niacycin, efficiently attenuates the *fsr* quorum-sensing system in sublethal concentrations (18). In the present study, we screened secondary fungal metabolites using the same screening system and found that ambucic acid, a known antifungal compound, inhibits the *fsr* quorum-sensing system through the inhibition of GBAP biosynthesis. We also demonstrated that ambucic acid inhibits the biosynthesis of cyclic peptide quorone of *Staphylococcus aureus* and *Listeria innocua*. This suggests the potential application of ambucic acid as an antipathogenic compound to target the quorum-sensing system of gram-positive bacteria.

**MATERIALS AND METHODS**

**Bacterial strains, media, and culture conditions.** *E. faecalis* strain OG1RF was used as a standard gelatinase-positive strain (3). *E. faecalis* US105 is a GBAP-negative but GBAP-sensitive strain used for the GBAP assay (18). All *E. faecalis* strains were cultured in Todd-Hewitt broth (THB) medium (Oxoid, Hampshire, United Kingdom) at 37°C with gentle agitation. The US105 strain (pOU2200 and pOU2301) was cultured in the presence of chloramphenicol (20 μg/ml) and erythromycin (50 μg/ml). *Staphylococcus aureus* 12600T and *S. aureus* 8325-4 (pSB2035) (25) were cultured aerobically in TSB-YE medium (30 g of tryptic soy broth and 6 g of yeast extract per liter) at 30°C or Luria-Bertani broth (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter) containing 7 μg/ml of chloramphenicol at 37°C, respectively. *Listeria innocua* ATCC 33907T was cultured in a chemically defined medium (9) at 30°C with shaking.

**Culture of fungi.** Fifty-one fungal strains were cultured for the screening. These strains were obtained from the Biotechnology Research Center at Toyama Prefectural University. Each strain was incubated by static culture at 25°C for approximately 1 month in three different media, namely, A-3-M (21), potato dextrose agar (39 g/liter) (Difco, Detroit, MI), and M-1-D medium (24). The culture broths were mixed with equal volumes of butanol and shaken for 2 h at room temperature. After centrifugation at 6,500 × *g* for 20 min, 1 ml of butanol was collected and evaporated to dryness in vacuo. The extract was stored at −20°C in a freezer until the screening assay. For the screenings, the butanol extract (1 ml) was dissolved in 25 μl of methanol, and 5 μl of this solution was added to 0.5 ml of fresh THB medium for the assays of gelatinase production or GBAP production.

For the large-scale production of ambucic acid, the fungal strain KAP-21 was incubated by static culture in A-3-M medium for approximately 1 month.

**First screening (assay for inhibition of gelatinase production in *E. faecalis* OG1RF).** The assay for the inhibition of gelatinase production in *E. faecalis* was performed using a method described previously (18). A culture (3 μl) of *E. faecalis* OG1RF grown overnight was inoculated into 0.5 ml of fresh THB medium containing the tested sample and was cultured for 5 h at 37°C. The culture supernatant was then collected after centrifugation at 9,100 × *g* for 5 min, and 25 μl of this culture supernatant was subjected to the gelatinase assay using azocoll as a substrate as described previously (15, 18). Briefly, 25 μl of *E. faecalis* culture supernatant was added to 0.5 ml of azocoll suspension, incubated for 4 h with constant mixing (170 rpm), and centrifuged at 20,000 × *g* for 5 min, and the optical density at 540 nm (OD540) of the supernatant was then measured.

**Second screening (assay for the inhibition of GBAP production in *E. faecalis* OG1RF).** The assay for the inhibition of GBAP production in *E. faecalis* was performed using a method described previously (18). A culture (3 μl) of *E. faecalis* OG1RF grown overnight was inoculated into 0.5 ml of fresh THB medium containing the tested sample. After 5 h of cultivation, the cells were removed from the culture by centrifugation at 10,000 × *g* for 5 min; the supernatant was then treated through a cellulose acetate filter (0.2 μm) (DISMIC-13CP; Advantec Toyo, Tokyo, Japan), and 100 μl of the filtrate was added to 400 μl of fresh THB medium. A culture (3 μl) of *E. faecalis* US105 grown overnight was inoculated into the conditioned medium and cultured for 5 h. The culture supernatant was then subjected to the gelatinase assay, and the GBAP activity was represented by the induced gelatine activity (OD540).

**Purification of ambucic acid.** The butanol extract of the KAP-21 culture (2 liters) was dissolved in 50 ml of 20% (vol/vol) acetonitrile and then applied onto a Sep-Pak Vac C18 cartridge column (35 ml, 10 g; Waters, Milford, MA). After washing with 100 ml of 20% acetonitrile containing 0.1% (vol/vol) trifluoroacetic acid (TFA), the column was eluted with 100 ml of 40%, 60%, and 80% acetonitrile containing 0.1% TFA. The fraction of 60% acetonitrile was evaporated, lyophilized, and redissolved in 3 ml of 30% acetonitrile containing 0.1% TFA. The solution was divided into six aliquots, and each aliquot was subjected to reverse-phase high-performance liquid chromatography (InertSil ODS-3, 20 by 150 mm; GL Sciences Inc., Tokyo, Japan). The column was washed with 20% acetonitrile in 0.1% TFA for 5 min at 10 ml/min and then developed by a gradient of 20% to 80% acetonitrile in 0.1% TFA for 30 min at the same flow rate. A single peak obtained at 27 min was collected, evaporated, and lyophilized. Fractionation of ambucic acid was obtained in the form of white powder and was confirmed to possess inhibitory activity against gelatinase production discussed in the above-mentioned assay.

**Mass spectrometry.** The high-performance liquid chromatography-purified fraction of ambucic acid was loaded onto an electrospray ionization-time of flight mass spectrometer (Accutof T100LC; Jeol, Tokyo, Japan). The following observations were recorded: the spectrum was obtained in the positive-polarity mode with constant mixing (170 rpm), and centrifuged at 20,000 × *g* for 5 min, and the chemical shifts were referenced to the residual solvent signals (δH = 3.31; δC = 49.8).

**NMR spectroscopy.** Nuclear magnetic resonance (NMR) spectra were measured using a Bruker Avance 500 spectrometer at 25°C. The purified ambucic acid (1.1 mg) was dissolved in 490 μl of deuterated methanol. The chemical shifts were referenced to the residual solvent signals (δH = 3.31; δC = 49.8).

**Effect of ambucic acid on GBAP signaling.** To examine the effects of ambucic acid on GBAP signaling, *E. faecalis* OU510 was cultured in the presence of GBAP and ambucic acid. A culture of *E. faecalis* OU510 (3 μl) grown overnight was inoculated into 0.5 ml THB containing 100 μM ambucic acid, and synthetic GBAP was added at various concentrations; the bacteria were cultured for 5 h. The culture supernatant was then subjected to the gelatinase assay.

**Effect of ambucic acid on GBAP processing.** The GBAP processing activity in the conditioned supernatant was analyzed by using primers 8048-f (5′-CTGAGGGCACTCGGCTACG-3′), 8048-rhis6 (5′-CCAAAAAGGATTGAAAAACCATCATCATCATCATGAGAGAATCG-3′), and pOU2300 (17) as a template. The PCR product was digested by Sfeli and then sequenced.
self-ligated using a DNA ligation kit (Takara, Kyoto, Japan). The resultant plasmid, pQU2301, was introduced into strain OU510, which already contains plasmid pQU2200 (17), by electroporation, and a transformant was selected on THB agar medium containing erythromycin (50 μg/ml) and chloramphenicol (20 μg/ml).

In order to examine the inhibitory effects of ambuc acid on GBAP biosynthesis, 20 μl of culture supernatant of each bacterial strain was analyzed by liquid chromatography-mass spectrometry (LC/MS). A culture of S. aureus 12600T (20 μl) grown overnight was inoculated into 2 ml of TSB-YE medium and cultured for 15 h. After cells were removed by centrifugation at 13,000 × g for 5 min, the culture supernatant was filtered (0.45 μm filter, and 40 μl) of this solution was applied for LC/MS as mentioned above, in addition to the one-dimensional NMR spectra, a series of two-dimensional NMR experiments agreed with the chemical structure of ambuc acid, 200 μM of ambuc acid was added to the washed cells in PBS, and the fluorescence was monitored for 15 min.

LC/MS. In order to monitor the biosynthesis of cyclic peptide quromones, the culture supernatant of each bacterial strain was analyzed by liquid chromatography/mass spectrometry (LC/MS). A culture of S. aureus 12600T (20 μl) grown overnight was inoculated into 2 ml of TSB-YE medium and cultured for 15 h. After cells were removed by centrifugation at 13,000 × g for 5 min, the culture supernatant was filtered using a 0.22-μm filter, and 40 μl of the filtrate was applied onto an LC/MS system (Agilent HP1100 liquid chromatograph, Agilent Zorbax Extend-C18 column by 50-mm column, and Agilent Acquity UPLC mass spectrometer). The elution from the MS analysis was performed under the following conditions: positive polarity, capillary temperature of 260°C, needle voltage of 2.0 kV, orifice voltage of 70 V, and ring lens voltage of 10 V. The eluate was directly loaded into the electrospray ionization-time of flight mass spectrometer. Mass analyses were performed under the following conditions: positive polarity, capillary temperature of 260°C, needle voltage of 2.0 kV, orifice voltage of 70 V, and ring lens voltage of 10 V. After scanning for molecular ions derived from column eluates in the m/z range of 100 to 2,000, an extracted ion chromatogram was plotted with detector counts in the mass range m/z 961 to 962. A culture of listeria innocua (25 μl) grown overnight was inoculated into 5 ml of chemically defined medium and cultured for 15 h. After the cells were removed by centrifugation at 12,000 × g for 5 min, the culture supernatant was filtered, and the culture filtrate was loaded onto a Sep-Pak Plus C18 cartridge column (360 mg; Waters), washed with 5 ml of 10% acetonitrile containing 0.1% TFA, and then eluted with 2 ml of 60% acetonitrile containing 0.1% TFA. The eluate was evaporated to dryness and then resuspended in 100 μl of 30% acetonitrile and 40 μl of this solution was applied for LC/MS as mentioned above, except that the gradient was from 10% to 36% acetonitrile in 0.05% aqueous TFA solution for 40 min. The monitor m/z 21 was selected from 699 to 700 and from 827 to 828.

Inhibitory effect of ambuc acid on hemolysis production in S. aureus. Various concentrations of ambuc acid in 50% acetonitrile (5 μl) were spotted onto a sheep blood agar plate (Kohjinbio, Osaka, Japan), and the solvent was dried under atmospheric pressure. The culture of S. aureus 12600T grown overnight was then diluted a thousandfold with fresh TSB-YE medium, and 0.2 μl of this diluted solution was spotted onto the agar plate. The plate was incubated at 37°C, and the zone of clearance surrounding the spots was observed and photographed after 16 h, 24 h, and 48 h.

RESULTS

Screening of inhibitors targeting for quorum sensing from fungal culture supernatants. Fifty-one strains from our fungal culture collection were cultured in three different media. The butanol extracts of 153 cultures were subjected to the first and second screenings. In the first screening, the standard gelatinase-producing strain, namely, E. faecalis OG1RF, was cultured in the medium containing the fungal butanol extract, and the gelatinase production of the strain was examined. Fourteen extracts showed significant inhibitory effects on gelatinase production without a strong inhibition of bacterial growth. In the second screening, E. faecalis OG1RF was cultured in a similar manner. GBAP activity in the culture supernatant was examined using E. faecalis OU510 as an indicator strain; this strain lacks GBAP biosynthesis but can produce gelatinase in response to GBAP. All 14 samples showed inhibitory effects on GBAP production. A butanol extract of an unidentified fungus, KAP-21, cultured in A-3 M medium showed clear and stable inhibitory activity and was eventually selected for further studies.

Identification of KAP-21A. The inhibitor produced by KAP-21, temporarily termed KAP-21A, was purified and subjected to structural analysis. Mass spectrometric results indicated that the molecular mass of KAP-21A was 350 kDa. 13C NMR spectra with a proton-decoupling pulse sequence or distortionless enhancement by polarization transfer sequence at a pulse angle of 135° indicated two methyl, six methylene, five methine, and six quaternary carbon atoms. According to chemical shifts in these spectra, the presence of four hydroxy methines, two olefinic carbons, one ketone, and one carboxyl group was suggested. Thus, we estimated the molecular formula to be C19H27O7, which corresponded to a molecular mass of 368 kDa. The difference between the estimated molecular mass and that observed from the mass spectrum suggested one dehydration and the molecular formula to be C18H26O6. In addition to the one-dimensional NMR spectra, a series of two-dimensional NMR analyses, namely, 1H-1H double-quantum-filtered correlation spectroscopy, 1H-13C total correlation spectroscopy, 13C-1H heteronuclear single quantum coherence spectroscopy, and 13C-1H heteromolecular multiple bond connectivity (HMBC), were performed. By analysis of these spectra coupled with the database search in SciFinder Scholar (American Chemical Society, Washington, DC) using the molecular formula, KAP-21A was revealed to be ambuc acid, which is a known antifungal compound. The results of proton and 13C assignment are shown in Table 1. The chemical shifts of all protons were almost identical to those of ambuc acid (11), and the resonances observed in the series of two-dimensional NMR experiments agreed with the chemical structure of ambuc acid (Fig. 1).

Mode of action of ambuc acid. Fig. 2 shows the effect of various concentrations of purified ambuc acid on the growth and gelatinase production of E. faecalis OG1RF. Ambuc acid had a 50% inhibitory concentration of approximately 10 μM and inhibited the production of gelatinase, but it did not show...
a marked inhibitory effect on the growth of \textit{E. faecalis} OG1RF in the tested concentration range lower than 1 mM.

In order to examine the effect of ambuic acid on GBAP signaling, GBAP-negative strain OU510 was cultured with GBAP at various concentrations and in the presence or absence of ambuic acid, and the induced gelatinase activity was measured (Fig. 3). The gelatinase induction was not found to have been inhibited by ambuic acid. This suggests that ambuic acid does not inhibit GBAP signaling but rather inhibits GBAP biosynthesis.

The effect of ambuic acid on GBAP biosynthesis was further investigated at the peptide level. Hexahistidine-tagged FsrD (His$_6$-FsrD) was expressed with or without FsrB'. In the absence of FsrB', His$_6$-FsrD was detected by the Western blotting technique using an anti-histidine tag antibody, while the His$_6$-FsrD band disappeared upon expression with FsrB' (Fig. 4A). This suggested that the proteolytic processing of His$_6$-FsrD was performed by FsrB'. However, when cultured with ambuic acid, this proteolytic processing as well as the GBAP activity were clearly inhibited (Fig. 4B).

**Inhibitory effect of ambuic acid on \textit{agr} expression in \textit{S. aureus}**. RNA III is the pivotal effector molecule in the \textit{agr} regulon acting primarily at the level of gene transcription. pSB2035 is a dual-reporter plasmid carrying the GFP gene (\textit{gfp}) and the bacterial luciferase gene (\textit{lux}) under an RNA III promoter (P3), allowing the in vivo measurement of \textit{agr} expression (25). When pSB2035 was introduced into an \textit{agr}+ strain, bioluminescence and fluorescence were observed in response to endogenous quormone (25). By using the reporter strain \textit{S. aureus} 8325-4(pSB2035), the effect of ambuic acid on \textit{agr} quorum sensing was investigated. Ambuic acid did not show a significant direct inhibitory effect on GFP fluorescence (Fig. 5, inset). Ambuic acid inhibited expressions of both GFP (Fig. 5) and luciferase (data not shown) at the same inhibitory concentrations as those observed for \textit{E. faecalis} quorum sensing. This suggested that ambuic acid was also effective on \textit{agr} quorum sensing in \textit{S. aureus}.

**Inhibitory effect of ambuic acid on biosynthesis of cyclic peptide quormone of other gram-positive bacteria**. In order to examine if the \textit{agr} inhibition of ambuic acid was caused by the inhibition of quormone biosynthesis, quormone production was investigated in \textit{S. aureus} cells cultured with ambuic acid. The cyclic peptide quormone of \textit{S. aureus} 12600T, namely, AIP-I, was clearly detected by LC/MS analysis of the culture supernatant. However, the peak that corresponded to AIP-I disappeared when this strain was cultured with 50 \mu M ambuic acid.

---

**TABLE 1. Chemical shifts of $^{13}$C and $^1$H and HMBC of KAP-21A (ambuic acid)**

<table>
<thead>
<tr>
<th>Position</th>
<th>$\delta^{13}$C</th>
<th>$\delta^1$H</th>
<th>HMBC$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>170.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>130.56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>134.40 6.68 (t)</td>
<td>C-1, C-4, C-5, C-18</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>27.28 2.79 (m)</td>
<td>C-2, C-3, C-5, C-6, C-10</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>59.82 3.76 (d)</td>
<td>C-4, C-5, C-7, C-8</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>74.49 4.83 (s)</td>
<td>C-8, C-9</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>149.29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>131.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>194.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>121.33 6.14 (d)</td>
<td>C-8, C-10, C-13</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>138.77 5.85 (m)</td>
<td>C-9, C-13, C-14</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>4.39 (d)</td>
<td>C-7, C-8</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>6.14 (d)</td>
<td>C-8, C-10, C-13</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>1.45 (m)</td>
<td>C-12, C-13, C-15, C-16</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>1.35 (m)</td>
<td>C-12, C-13, C-14, C-16, C-17</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>1.35 (m)</td>
<td>C-12, C-13, C-14, C-16, C-17</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>0.90 (s)</td>
<td>C-15, C-16</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>1.87 (s)</td>
<td>C-1, C-2, C-3, C-5, C-6</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>4.59 (d), 4.51 (d)</td>
<td>C-7, C-8, C-9</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Position numbered as shown in Fig. 1.

$^b$ Carbon to which HMBC was observed.

---

**FIG. 1.** Structure of KAP-21A (ambuic acid) (11).

**FIG. 2.** Effect of various concentrations of ambuic acid on the cell growth and gelatinase production of \textit{E. faecalis} OG1RF. \textit{E. faecalis} OG1RF was grown for 5 h in the presence of ambuic acid at the indicated concentrations; the cell density in the culture supernatant was measured at the OD$_{660}$ (open circles), and the gelatinase activity was measured at the OD$_{540}$ (closed circles), as described in Materials and Methods. The data are presented as averages ± standard deviations of experiments performed in duplicate.

**FIG. 3.** Effect of ambuic acid (100 \mu M) on gelatinase production in \textit{E. faecalis} OU510 induced by GBAP at various concentrations. \textit{E. faecalis} OU510 was grown for 5 h in the presence of the indicated concentrations of synthetic GBAP with (open circles) or without (closed circles) 100 \mu M ambuic acid. The gelatinase activity (OD$_{540}$) and growth (OD$_{660}$) in the culture supernatant were then measured in duplicate experiments. The value of gelatinase activity/growth was calculated for each experiment, and the average values were plotted. Standard deviations in all experiments were less than 0.1.
acid (Fig. 6A). This result indicated that ambuic acid inhibited the biosynthesis of AIP-1 in *S. aureus*.

The effect of ambuic acid was also examined with regard to quorum biosynthesis in *Listeria*. In the culture supernatant of *Listeria innocua* ATCC 33090T, two peaks corresponding to putative cyclic peptide quorumones, namely, LsrD698 and LsrD826, were detected at \( m/z \) 699 and 827, respectively. When cultured in the presence of 100 \( \mu \)M ambuic acid, these two peaks disappeared, suggesting that ambuic acid also inhibits the biosynthesis of cyclic peptide quorumones in *Listeria innocua* (Fig. 6B).

**FIG. 5.** Effect of ambuic acid on \( \text{agr} \) expression in *S. aureus* 8325-4(pSB2035). The strain was grown in the absence or presence of ambuic acid. After 7 h, the OD\(_{620}\) and GFP fluorescence (fluorescence at 490 nm) were measured. The experiment was done in triplicate, and averages were graphed with standard deviations. In order to examine the direct effect of ambuic acid on GFP activity, after harvesting cells that were cultured without ambuic acid, 200 \( \mu \)M ambuic acid was added to the washed cells in PBS, and the fluorescence was then monitored for 15 min (inset).

**FIG. 6.** Effect of ambuic acid on production of AIP-I in *S. aureus* 12600T (A) and LsrD698 and LsrD826 in *Listeria innocua* ATCC 33090T (B). (A) *S. aureus* 12600T was grown for 15 h, and the culture filtrate was applied for LC/MS analysis. The liquid chromatography eluate was monitored at \( m/z \) 961, which corresponds to the molecular ion of AIP-I (B). *Listeria innocua* ATCC 33090T was grown for 15 h, and the partially purified culture filtrate was applied for LC/MS analysis. The liquid chromatography eluate was monitored at \( m/z \) 699 and \( m/z \) 827, which corresponded to the molecular ions of LsrD698 and LsrD826, respectively.

**Influence of ambuic acid on hemolysin production in *S. aureus*.** It is well known that hemolysin production is regulated by the \( \text{agr} \) quorum-sensing system in staphylococci. Figure 7 shows the different concentrations of ambuic acid solution that were spotted onto sheep blood agar, and hemolysin-producing *S. aureus* 12600T was then inoculated onto each site. In the control, at 16 h after inoculation, a hemolytic zone (Fig. 7, white ring) around the bacterial colony was observed. The area of the hemolytic zone became smaller with an increase in the concentration of ambuic acid, and almost no hemolytic zone was observed on the site spotted with 5 \( \mu \)l of 20 mM ambuic acid, while the size of the colony did not differ from that of the control. The inhibitory effect of ambuic acid gradually decreased with time until 48 h, as shown in Fig. 7.
GBAP-mediated gelatinase biosynthesis in *E. faecalis* but also AIP-mediated hemolysin production in *S. aureus*. However, even at high concentrations, the inhibitory effect was not substantial or sustainable. It would be necessary and possible to develop more potent inhibitors based on ambucic acid as a lead compound.

**ACKNOWLEDGMENTS**

This work was supported in part by grants-in-aid for scientific research from the Japan Society for the Promotion of Science (grant no. 17580068 and 19658034); the Ministry of Education, Culture, Sports, Science, and Technology of Japan (grant no. 16087203); the Kato Memorial Bioscience Foundation; and the Waksman Foundation of Japan.

We thank Paul Williams at University of Nottingham for providing *S. aureus* 8325-4(pSB2035).

**REFERENCES**


