Antibacterial Activity of Stilbene Oligomers against Vancomycin-Resistant Enterococci (VRE) and Methicillin-Resistant *Staphylococcus aureus* (MRSA) and Their Synergism with Antibiotics

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Two resveratrol trimers, gnemonol B isolated from *Gnetum gnemon* and gnetin E obtained from the Gnetum species, were found to exhibit strong antibacterial activities against vancomycin-resistant Enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA). The MIC values of gnemonol B against five strains of VRE and nine strains of MRSA were 12.5 and 6.25 μg/ml, respectively. The MIC values of gnetin E against five strains of VRE and nine strains of MRSA ranged from 12.5 to 25 μg/ml. These compounds also showed synergistic effects when used in combination with commercially available antibiotics according to the evaluation method using FIC indices. These findings suggested that the application of the test compounds alone or in combination with antibiotics might be useful in controlling and treating VRE and MRSA infections.

Key words: Antibacterial Activity/Stilbene Oligomers/VRE/MRSA/Synergism.

INTRODUCTION

As Enterococci and *Staphylococcus aureus* are two of the leading causes of nosocomial infections in long term healthcare facilities, reports on vancomycin-resistant Enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) infections in hospitals have been increasing worldwide (Emori and Gaynes, 1993; Leclercq and Courvalin, 1997; Barie, 1998; Murry, 1997; Molllering, 1998; Cal et al., 2004; Bartley et al., 2001; Nagayama, 2006).

In recent years, there have been a number of reports on useful trials carried out to control the infections caused by VRE (Garner, 1996; Slaughter et al., 1996; Montecalvo et al., 1999; Nourse et al., 2000; Hanna et al., 2001; Montecalvo et al., 2001; Shaikh et al., 2002; Calfee et al., 2003; Warren et al., 2004; Mascini and Bonten, 2005; Zoutman and Ford, 2005) and MRSA (Voss et al., 1994; Coolson, 1995; Cox et al., 1995; Working Party Report, 1998; Kotilainen et al., 2001; Hori, 2002; Bissett, 2005; Schelenz et al., 2005).

However, further trials would be necessary to find more reliable methods to control the VRE and MRSA infections adequately. In this context, the use of natural products as anti-VRE and anti-MRSA agents would be a promising area of investigation leading towards the eventual prevention and treatment of VRE and MRSA infections. Furthermore, it would be very important to investigate the synergistic activities of the active natural products and commercially available antibiotics, with the hope of enhancing their activity.

Recently the anti-MRSA activity of natural products such as flavonoids, for example, sophoraflavanone G...
(Sato et al., 1995), xanthones such as rubraxanthone (linuma et al., 1996), and oligostilbenes such as hemsleyanol D and gnetin E (Nitta et al., 2002) have been reported.

Resveratrol, one of the stilbene derivatives, is well known as one of the polyphenol phytoalexins found in grapes and wine, and is a strong chemopreventive agent with promising safety records with regard to human consumption and unique forms of cell death induction in a variety of tumor cells (Mohan et al., 2006).

There have been some reports about the antibacterial activity of stilbene (Atef et al., 1979; Inamori et al., 1991; Wyrzykiewicz et al., 1994; Wyrzykiewicz et al., 2000; Venkateswarlu et al., 2002), but none about the antibacterial activity of stilbene oligomers against bacteria including VRE and MRSA.

In the present experiment we tested the anti-VRE and anti-MRSA activities of 37 stilbene oligomers isolated from Gnetaceous plants (Iliya et al., 2002a, 2002b). In addition, their synergistic activities with the commercially available antibiotics such as ampicillin (ABPC), gentamicin (GM), minocycline (MINO), fosfomycin (FOM) and vancomycin hydrochloride (VCM) were also investigated.

MATERIALS AND METHODS

Stilbene oligomers
Among the stilbene oligomers isolated from Gnetum species (G. africanum, G. gnemon and G. gnemonoides), 37 compounds shown in Table 1 were used in the present experiment.

Antibiotics
Ampicillin (ABPC), gentamicin (GM), minocycline (MINO), fosfomycin (FOM) and vancomycin hydrochloride (VCM) were used for the synergistic studies.

Tested bacteria
VRE: Five strains of VRE (Enterococcus faecalis ATCC 51299, E. faecalis ATCC 51575, E. faecium ATCC 51559, E. faecium KIHC-237 and E. gallinarum KIHC-241) were used in this experiment. The three ATCC strains were purchased from the American Type Culture Collection (ATCC). Two strains of E. faecium KIHC-237 and E. gallinarum KIHC-241 were supplied by the Kobe Institute of Public Health. The genotypes of E. faecalis ATCC 51299, E. faecium KIHC-237 and E. gallinarum KIHC-241 are van B(+), van A(+) and van C1(+), respectively. The genotypes of the other two VRE, E. faecalis ATCC 51575 and E. faecium ATCC 51559 were unknown.

VSE: Three strains of vancomycin-sensitive Enterococci (VSE) (E. faecalis IFO 12965, E. faecium IFO 3535 and E. faecalis ATCC 8459) were used in this experiment. The strains were purchased from Institute for Fermentation of Osaka (IFO), Japan, and ATCC, respectively.

MRSA: Three strains each (total: nine strains) of methicillin-resistant Staphylococcus aureus (MRSA) were kindly donated by the Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka National Hospital and Kitano Hospital in 1997.

MSSA: Methicillin-sensitive Staphylococcus aureus (MSSA), Staphylococcus aureus IFO 13276, S. aureus IFO 12732 and S. aureus IFO 3060 used in this experiment were purchased from IFO.

Broth
SCD broth (Nihon Pharm. Co., Ltd.) was used for pre-incubation of VRE, VSE, MRSA and MSSA. Mueller-Hinton (MH) Agar (Difco Co., Ltd.) was used for the measurement of MIC.

MIC
MIC values were determined by the agar dilution method (Goto et al., 1981) using a micro-inoculater (Sakuma Seisakusho Co., Ltd., Tokyo).

MIC of the above five strains of VRE and three strains of VCM were measured at 250, 32, 200, 200 and 16, μg/ml, while MIC values of the nine strains of MRSA and three strains of methicillin (DMPPC) were measured at 12.5, 400, 25, 12.5, 400, 1600, 25, 12.5 and 400, μg/ml, respectively.

Synergism of gnemonol B and gnetin E with the commercially available antibiotics
Solutions (50 % dimethylsulfoxide solution) of gnemonol B and gnetin E (see Fig.1) were prepared separately. A solution of gnemonol B or gnetin E in combination with each designated antibiotic was prepared by the doubling dilution method with sterilized water, and each solution was poured into sterilized plastic petri dishes separately. Sterilized MH agar 8 ml (MH agar 9 ml was then poured into gnemonol B and gnetin E alone or the antibiotic alone) was poured into the above petri dishes and mixed. After cooling, the MICs of gnemonol B and gnetin E alone, the antibiotics alone, and their combinations, were examined. The fraction inhibitory concentration (FIC) indices were calculated by the method of Didry et al. (1993), and the interactive effects between the test stilbene oligomers (gnemonol B and gnetin E) and the antibiotics were examined.
TABLE 1. Stilbene oligomers (37 compounds) tested in the present experiment.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Plant</th>
<th>Profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>gnemonol A</td>
<td>Gnetum gnemon</td>
<td>trimer</td>
</tr>
<tr>
<td>latifolol</td>
<td>Gnetum gnemon</td>
<td>trimer</td>
</tr>
<tr>
<td>gnemonol B</td>
<td>Gnetum gnemon</td>
<td>tetramer</td>
</tr>
<tr>
<td>gnemonol G</td>
<td>Gnetum gnemon</td>
<td>dimer</td>
</tr>
<tr>
<td>gnetoflavanol F</td>
<td>Gnetum gnemon</td>
<td>flavonostilbene</td>
</tr>
<tr>
<td>gnemonol J</td>
<td>Gnetum gnemon</td>
<td>trimer</td>
</tr>
<tr>
<td>gnemonol D</td>
<td>Gnetum gnemon</td>
<td>trimer</td>
</tr>
<tr>
<td>gnetoflavanol E</td>
<td>Gnetum gnemon</td>
<td>flavonostilbene</td>
</tr>
<tr>
<td>gnemonoside K</td>
<td>Gnetum gnemon</td>
<td>trimer</td>
</tr>
<tr>
<td>gnemonol I</td>
<td>Gnetum gnemon</td>
<td>trimer</td>
</tr>
<tr>
<td>gnemonoside F</td>
<td>Gnetum gnemonoids</td>
<td>trimeric-glicoside</td>
</tr>
<tr>
<td>gnetin D</td>
<td>Gnetum gnemonoids</td>
<td>dimer</td>
</tr>
<tr>
<td>gnemonol C</td>
<td>Gnetum gnemonoids</td>
<td>tetramer</td>
</tr>
<tr>
<td>gnetin C</td>
<td>Gnetum gnemonoids</td>
<td>dimer</td>
</tr>
<tr>
<td>gnemonoside B</td>
<td>Gnetum gnemonoids</td>
<td>dimer</td>
</tr>
<tr>
<td>gnemonoside A</td>
<td>Gnetum gnemonoids</td>
<td>dimer</td>
</tr>
<tr>
<td>gnetulina</td>
<td>Gnetum parvifolium</td>
<td>dimer</td>
</tr>
<tr>
<td>unknown</td>
<td>Welwitschia mirabilis</td>
<td>trimeric-glicoside</td>
</tr>
<tr>
<td>parvifolol A</td>
<td>Gnetum parvifolium</td>
<td>dimer</td>
</tr>
<tr>
<td>2b-OH-ampelopsin F</td>
<td>Gnetum parvifolium</td>
<td>dimer</td>
</tr>
<tr>
<td>e-viviferin</td>
<td>Gnetum africanum</td>
<td>dimer</td>
</tr>
<tr>
<td>res-diglicoside</td>
<td>Gnetum africanum</td>
<td>monomeric-glicoside</td>
</tr>
<tr>
<td>isorha-monoglicoside</td>
<td>Gnetum africanum</td>
<td>monomeric-glicoside</td>
</tr>
<tr>
<td>gneafraicanin A</td>
<td>Gnetum africanum</td>
<td>dimer</td>
</tr>
<tr>
<td>isorha-diglicoside</td>
<td>Gnetum africanum</td>
<td>monomeric-glicoside</td>
</tr>
<tr>
<td>res-monoglicoside</td>
<td>Gnetum africanum</td>
<td>monomeric-glicoside</td>
</tr>
<tr>
<td>gneafraicanin C</td>
<td>Gnetum africanum</td>
<td>dimer</td>
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<tr>
<td>scirpusin A</td>
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<td>dimer</td>
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<td>Gnetum africanum</td>
<td>dimer</td>
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<td>Gnetum africanum</td>
<td>trimer</td>
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<td>gnetin E</td>
<td>Gnetum africanum</td>
<td>dimer</td>
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<tr>
<td>gneafraicanin A</td>
<td>Gnetum africanum</td>
<td>dimer</td>
</tr>
<tr>
<td>biisorhapontigenin A</td>
<td>Gnetum africanum</td>
<td>dimer</td>
</tr>
<tr>
<td>longusol A</td>
<td>Gnetum africanum</td>
<td>dimer</td>
</tr>
<tr>
<td>gnetoflavanol A</td>
<td>Gnetum africanum</td>
<td>flavonostilbene</td>
</tr>
<tr>
<td>amphiopsin E(trans)</td>
<td>Gnetum gnemon</td>
<td>trimer</td>
</tr>
</tbody>
</table>

FIG. 1. Structural formula of gnemonol B and gnetin E.
TABLE 2. MIC values of gnemonol B and gnetin E against 5 strains of VRE and 3 strains of VSE.

<table>
<thead>
<tr>
<th>MIC (µg/mL)</th>
<th>Gnemonol B</th>
<th>Gnetin E</th>
<th>Gentamicin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enterococcus faecalis ATCC 51299 (VRE)</strong></td>
<td>12.5</td>
<td>12.5</td>
<td>&gt;100</td>
</tr>
<tr>
<td><strong>Enterococcus faecalis ATCC 51575 (VRE)</strong></td>
<td>12.5</td>
<td>12.5</td>
<td>&gt;100</td>
</tr>
<tr>
<td><strong>Enterococcus faecium ATCC 51559 (VRE)</strong></td>
<td>12.5</td>
<td>12.5</td>
<td>6.25</td>
</tr>
<tr>
<td><strong>Enterococcus faecium KIH-237 (VRE)</strong></td>
<td>12.5</td>
<td>25</td>
<td>6.25</td>
</tr>
<tr>
<td><strong>Enterococcus gallinarum KIH-241 (VRE)</strong></td>
<td>12.5</td>
<td>25</td>
<td>3.13</td>
</tr>
<tr>
<td><strong>Enterococcus faecalis IFO 12965 (VSE)</strong></td>
<td>12.5</td>
<td>25</td>
<td>12.5</td>
</tr>
<tr>
<td><strong>Enterococcus faecium IFO 3535 (VSE)</strong></td>
<td>12.5</td>
<td>25</td>
<td>6.25</td>
</tr>
<tr>
<td><strong>Enterococcus faecalis ATCC 8459 (VSE)</strong></td>
<td>12.5</td>
<td>25</td>
<td>6.25</td>
</tr>
</tbody>
</table>

a) Purchased from the American Type culture Collection (ATCC).
b) Supplied by the Kobe Institute of Public Health.
c) Purchased from the Institute for Fermentation of Osaka (IFO), Japan.

RESULTS

Antibacterial activities of gnemonol B and gnetin E against VRE, MRSA, VSE and MSSA

GM, one of the representative antibiotics, was used as the comparative standard.

Gnemonol B was found to be active against five strains of VRE and nine strains of MRSA with MIC values of 12.5 and 6.25 µg/ml, respectively. Gnemonol B was also active against the three strains of VSE and MSSA with MIC values of 12.5 and 6.25 µg/ml, respectively. Gnetin E also exhibited the antibacterial activities against five strains of VRE, nine strains of MRSA and three strains of VSE and MSSA with MIC values ranging from 12.5 and 25 µg/ml. The effects of gnetin E on MRSA in the present study were roughly similar to ones earlier mentioned (Slaughter et al., 1996). The activity of gnetin E was weaker than gnemonol B (Tables 2 and 3).

No anti-VRE and anti-MRSA activities in the other test stilbene oligomers (35 samples) were found.

Synergism between gnemonol B or gnetin E and commercially available antibiotics against VRE, MRSA, VSE and MSSA

We investigated the synergistic effect by using gnemonol B and gnetin E. Gnemonol B exhibited a partial synergistic effect in combination with ABPC, GM, MINO, FOM and VCM against VRE. It also showed partial synergism with GM against MRSA. The average of FIC indices against VRE were calculated as 0.703±0.105, 0.550±0.068, 0.678±0.106, 0.763±0.155 and 0.624±0.169. The FIC indices of gnemonol B with ABPC, GM, MINO, FOM and VCM against MRSA were also calculated as 1.323±0.332, 0.918±0.306, 1.085±0.124, 1.089±0.121 and 1.403±0.285, respectively (Fig. 2).

In the combination of gnemonol B with ABPC, GM, MINO, FOM or VCM, partial synergism against VSE was exhibited, and the FIC indices were calculated as 0.708±0.072, 0.501±0.249, 0.833±0.144, 0.750±0.000 and 0.750±0.000, respectively.

Synergism of gnemonol B with GM was observed against MSSA (FIC index: 0.719±0.248). FIC indices of the other antibiotics such as ABPC, MINO, FOM and VCM were calculated as 1.250±0.250, 0.719±0.248, 1.188±0.108, 1.172±0.135 and 1.500±0.000 (Fig. 2).

Partial synergism of gnetin E with GM and VCM against VRE, and that of gnetin E with GM, MINO, FOM and VCM against MRSA were respectively observed. The FIC indices of gnetin E in combination
FIG. 2. Synergism between gemonol B and the commercially available antibiotics against five strains of vancomycin-resistant Enterococci (VRE), three strains of vancomycin-sensitive Enterococci (VSE), nine strains of methicillin-resistant Staphylococcus aureus (MRSA) and three strains of methicillin-sensitive Staphylococcus aureus (MSSA).

*FIC index indicates the average values and standard deviation.
FIC index ≤ 0.5: Synergistic effect
0.5 < FIC index < 1.0: Partially synergistic effect
1.0 ≤ FIC index: No synergistic effect
FIC index ≥ 2.0: Antagonistic effect

**Antibiotics tested are as follows: ABPC: Ampicillin, GM: Gentamicin, MINO: Minocycline, VCM: Vancomycin Hydrochloride

with GM and with VCM against VRE were 0.770 ± 0.229 and 0.746 ± 0.248, respectively. The FIC indices of gnetin E in combination with ABPC, GM, MINO, FOM and VCM against MRSA were 1.195 ± 0.369, 0.667 ± 0.107, 0.913 ± 0.317, 0.809 ± 0.264 and 0.854 ± 0.203, respectively.

Partial synergism of gnetin E in combination with ABPC, GM and FOM against VSE was observed, and the FIC indices were 0.705 ± 0.261, 0.750 ± 0.000 and 0.698 ± 0.289 (Fig.3).

Against MSSA, the FIC indices of gnetin E in combination with ABPC, GM, MINO, FOM and VCM were 1.667 ± 0.577, 0.594 ± 0.054, 1.333 ± 0.144, 1.130 ± 0.117 and 1.083 ± 0.289, respectively (Fig.3).

DISCUSSION

Among many stilbene oligomers the antitumor effect of resveratrol oligomers (Ito et al., 2003), and cytotoxic and antimutagenic effects on stilbenes (Kim et al., 2002) have already been investigated. In
FIG. 3. Synergism between gnetin E and commercially available antibiotics against five strains of vancomycin-resistant Enterococci (VRE), three strains of vancomycin-sensitive Enterococci (VSE), nine strains of methicillin-resistant Staphylococcus aureus (MRSA) and three strains of methicillin-sensitive Staphylococcus aureus (MSSA).

*FIC index indicates the average values and standard deviation.

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**Antibiotics tested are as follows: ABPC: Ampicillin, GM: Gentamicin, MINO: Minocycline, VCM: Vancomycin Hydrochloride

In the experiment of gnemonol B or gnetin E against gram negative bacteria such as Escherichia coli NBRC 3972, Proteus vulgaris NBRC 3988, Serratia marcescens NBRC 12648, Escherichia coli O157 (ATCC 43888), Klebsiella pneumoniae NBRC 13277 and Pseudomonas aeruginosa NBRC 13275, no activity was observed (data not shown). Synergism between gnemonol B or gnetin E and the commercially available antibiotics such as ABPC, GM, MINO, FOM and VCM was also investigated. In the present study gnemonol B showed partial synergism with ABPC, GM, MINO, FOM and VCM against VRE. Partial synergism was also found in the combination of gnemonol B with GM against MRSA. Furthermore, partial synergism of gnetin E with GM and VCM against VRE, and that of gnetin E with GM, MINO, FOM and VCM

In this paper, we investigated other possible activities of stilbene oligomers.

Gnemonol B isolated from Gnetaceous plants was found to be active against both VRE and MRSA with MIC values of 12.5 and 6.25 μg/ml, respectively. Although the activity of gnetin E against VRE and MRSA was weaker than gnemonol B, the MIC values were small and ranged between 12.5 and 25 μg/ml. In the experiment of gnemonol B or gnetin E against gram negative bacteria such as Escherichia coli NBRC 3972, Proteus vulgaris NBRC 3988, Serratia marcescens NBRC 12648, Escherichia coli O157 (ATCC 43888), Klebsiella pneumoniae NBRC 13277 and Pseudomonas aeruginosa NBRC 13275, no activity was observed (data not shown). Synergism between gnemonol B or gnetin E and the commercially available antibiotics such as ABPC, GM, MINO, FOM and VCM was also investigated. In the present study gnemonol B showed partial synergism with ABPC, GM, MINO, FOM and VCM against VRE. Partial synergism was also found in the combination of gnemonol B with GM against MRSA. Furthermore, partial synergism of gnetin E with GM and VCM against VRE, and that of gnetin E with GM, MINO, FOM and VCM
against VRE were also found. These activities of partial synergism were reconfirmed by our results obtained from the test described by Williamson (2001) (data not shown).

The use of phytoalexin (the antibacterial compound) isolated from the natural products would be considerably valuable for the controlling of infectious bacteria such as VRE and MRSA, etc. The main reason was as follows. No reports have been found about bacteria resistant to natural products including the tested stilbene oligomers (gnemonol B and gnetin E). The use of the antibiotics would be decreased because of the partial synergism between gnemonol B or gnetin E and the commercially available antibiotics, and the detection ratio of the resistant bacteria would become lower.

From the above mentioned results, these findings suggested that the use of gnemonol B or gnetin E alone or in combination with some antibiotics could be useful in controlling and treating VRE and MRSA infections. Further investigation will be performed using in vivo trials in the near future. Good results would be expected in such in vivo investigations of gnemonol B and gnetin E.

Recently we reported the anti-VRE and anti-MRSA activities of \( \alpha \)-mangostin isolated from the stem bark of *Garcinia mangostana* L. (Sakagami et al., 2005). In this report, we also found the strong anti-VRE and anti-MRSA active agents in natural products. These results might be valuable for the controlling of nosocomial infectious bacteria such as VRE and MRSA.

**REFERENCES**


