Anti-methicillin Resistant *Staphylococcus aureus* (MRSA) Compounds Isolated from *Laurus nobilis*

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Received March 7, 2008; accepted June 13, 2008; published online June 23, 2008

We found that an extract from *Laurus nobilis* L. (Lauraceae) leaves showed antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA). We purified two flavonoids as the effective compounds and identified them as kaempferol 3-*O*-α-L-(2″,4″-di-*E*-*p*-coumaroyl)-rhamnoside (C2) and kaempferol 3-*O*-α-L-(2″-*Z*-*p*-coumaroyl-4″-*E*-*p*-coumaroyl)-rhamnoside (C3). Both compounds showed strong antibacterial activity not only against MRSA but also against vancomycin-resistant enterococci (VRE). There was low or no antibacterial activity of C2 and C3 for *Streptococcus pneumoniae*, *Pseudomonas aeruginosa* and *Serratia marcescens*.

Key words methicillin-resistant *Staphylococcus aureus*; *Laurus nobilis*; vancomycin-resistant enterococci; anti-methicillin-resistant *Staphylococcus aureus* compound; anti-vancomycin-resistant enterococci

The incidence of infectious disease caused by drug resistant bacteria that are represented by methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE) and so on is extremely important in both community and hospital settings.[1)] Methicillin was introduced in Europe in 1959 and in the United States in 1961, and the first cases of MRSA were reported in the United Kingdom in 1961, followed soon thereafter by reports in Japan and Australia.[1)] So far, tremendous numbers of the MRSA strain have been reported in the world. Aucken *et al.* reported an epidemic comprised of MRSA, designated EMRSA-17, that showed resistances to multiple antibacterial agents including methicillin, ciprofloxacin, erythromycin, fusidic acid, rifampicin, gentamicin, kanamycin, neomycin, streptomycin and tetracycline.[2)] In general, MRSA strains show resistance against multiple antimicrobial agents although the ranges and extents of these resistances are versatile. In many years past, vancomycin was the only effective drug for serious MRSA infections. In 1996, *S. aureus* with intermediate resistance to vancomycin (VISA; MIC was 8 to 16 μg/ml) was first observed in a strain isolated from a hospitalized patient in Japan.[3)] Moreover, in 2002, a high-level vancomycin resistant *S. aureus* (VRSA: MIC was 1024 μg/ml) strain was isolated from a dialysis patient in the U.S.A.[4)] The report provided a genetic analysis in that the *vanA* gene made MRSA highly resistant to vancomycin. Up to the present day, four new agents with anti-MRSA activity have been introduced (quinupristin-dalfopristin, linezolid, daptomycin and tigecycline) in the U.S.A. But among them, only quinupristin-dalfopristin and linezolid are clinically available in Japan. As was concerned, resistance to linezolid has already emerged in clinical isolates of *S. aureus*.[5,6)]

VRE was not observed until the 1980s. During the 1990s, however, a drastic rise in VRE infections occurred. It has been reported that approximately 30% of all enterococci isolated from intensive care patients units are now resistant to vancomycin.[7)] Among the clinically isolated enterococcal species, 80—90% are *Enterococcus faecalis* and others are mainly *E. faecium*.[8)] Intrinsically, enterococci (especially *E. faecium*) possess a broad range of resistance against antimicrobials.

Under these circumstances, the demand for the development of new antibacterial agents effective against MRSA and/or VRE is a matter of great urgency. Thus, we have been trying to discover effective growth inhibitors against MRSA and/or VRE. There are several reports on natural anti-MRSA compounds, such as 3-arylcoumarines,[9)] licoricidin,[10)] cudraxanthone S,[11)] piperitylmagnolol,[12)] and isolupalbigenin.[13)] Here we report two anti-MRSA compounds, which showed stronger anti-MRSA activity than these compounds, isolated from leaves of *Laurus nobilis* (laurel), a kind of herb.

MATERIALS AND METHODS

Materials MRSA strains OM481, OM505, OM584, OM623 and N315 are clinically isolated strains. MRSA COL and MSSA (methicillin-sensitive *S. aureus*) 209P are laboratory collections. Leaves of *L. nobilis* (laurel) cultivated in Turkey were purchased from Toho TH2, Inc. (Kobe, Japan). Ciprofloxacin, erythromycin, norfloxacin oxacillin, tetracycline, vancomycin, isoquercitrin, kaempferol, *p*-coumaric acid, quercetin, quercitrin and rutin were from commercial sources.

Extraction and Purification of Effective Compounds Dried ground leaves (1.5 kg) of *L. nobilis* were homogenized in 70% acetone and filtered. The acetone extract was then concentrated and extracted with hexane, ethyl acetate, successively. The ethyl acetate extract (45 g) was subjected to column chromatography over DIAION HP-20 (Mitsubishi Kasei Co., Ltd.) and eluted with H_2O and aqueous methanol (60, 70, 80, 90, 100%) in a stepwise gradient manner. Thereafter, a 90% methanol fraction (3.5 g) which showed the highest anti-MRSA activity was subjected to a Sephadex LH-20 (Amersham Biosciences, Sweden) column, eluted with 100% ethanol, and fractionated into 8 fractions (Fraction A to H). The Fraction D (1.6 g) which showed the highest activ-

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ity was subjected to preparative TLC using Kieselgel 60F254 plates (0.2 mm thick, Merck) and developed with a $CHCl_3$–MeOH–HCOOH (9 : 1 : 1) solvent system. A zone showing the highest activity was collected (120 mg). The preparative HPLC was performed on an ODS-3 inertsil column (GL Science Inc.), eluted with MeOH–H_2O (70 : 30) and effective compounds C2 (27.4 mg) and C3 (62.4 mg) were obtained.

Susceptibility Testing Minimum inhibitory concentrations (MICs) of antimicrobial agents were determined by broth dilution techniques, according to the instructions of the Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS). The MIC determinations were made in triplicate on separate occasions. Broth MIC testing was performed in 96-well microtiter trays with an inoculum of about 10^5 CFU in 100 μl of Mueller–Hinton broth (Difco) supplemented with 0.85% NaCl. MIC values were obtained after incubation at 35 °C for 24 h. Compounds C2 and C3 were dissolved in MeOH before dilution into MHB for MIC determinations.

RESULTS

Purification and Identification of Effective Compounds We prepared methanol extracts from many plants and tested the antibacterial activities of these extracts. We found that an extract from the leaves of *Laurus nobilis* (laurel) showed strong anti-MRSA activity. We also found that the extract showed potent anti-VRE activity. We tried to purify the anti-MRSA compound(s) by using column chromatography with DIAION HP-20, Sephadex LH-20, thin-layer chromatography (TLC) and HPLC as described under Materials and Methods. We obtained two effective compounds, and identified them as kaempferol 3-*O*-α-L-(2″,4″-di-*E*-*p*-coumaroyl)-rhamnoside (C2) and kaempferol 3-*O*-α-L-(2″-*Z*-*p*-coumaroyl-4″-*E*-*p*-coumaroyl)-rhamnoside (C3) (Fig. 1) by using ^{1}H- and ^{13}C-NMR, UV–visible spectra, ESI-MS spectra, optical rotation.

Anti-MRSA Activities of C2 and C3 We measured the MICs of C2 and C3 with several MRSA strains. We observed MIC values of 1 to 2 μg/ml with the MRSA strains tested (Table 1). *S. aureus* OM481, OM505, OM584 and OM623 are clinically isolated MRSA strains that showed very high MICs of oxacillin, fluoroquinolones and erythromycin. Thus, these antimicrobial agents are basically ineffective on these MRSA strains. The MICs of these drugs and of tetracycline with COL and N315 MRSA strains were diverse. C2 and C3 were able to inhibit the growth of all of these MRSA strains at relatively low concentrations. MICs of C2 and C3 with these MRSA strains were a little higher than that of vancomycin (Table 1). The C2 and C3 compounds showed a similar but slightly lower MIC (0.5 μg/ml) with a methicillin-sensitive *S. aureus* 209P, an MSSA strain, than with the MRSA strains. Thus, C2 and C3 are effective on both MRSA and MSSA at similar concentrations.

Structure–Activity Relationship We tested the effects of compounds with structures similar or partially similar to the structures of C2 and C3 on MRSA and MSSA to get some insights into the structure–activity relationship. The C2 and C3 compounds consist of kaempferol, rhamnose, and coumaroyl moieties. Kaempferol and *p*-coumaric acid did not show anti-MRSA and anti-MSSA activities (Table 1), and rhamnose showed no growth inhibition (data not shown).

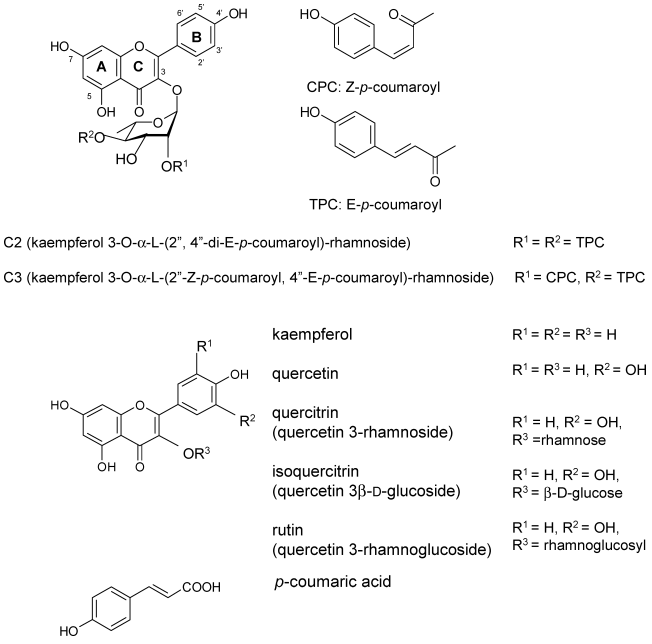

Fig. 1. Structures of C2, C3, and Related Compounds

Table 1. Antibacterial Activity of C2 and C3 on *S. aureus*

Compound	MIC (μg/ml)						
	OM481	OM505	OM584	OM623	COL	N315	209P
C2	1	1	2	2	1	1	0.5
C3	1	2	2	2	1	1	0.5
Kaempferol	>256	>256	>256	>256	>256	>256	>256
Quercetin	>256	>256	>256	>256	>256	>256	>256
Quercitrin	>256	>256	>256	>256	>256	>256	>256
Isoquercitrin	>256	>256	>256	>256	>256	>256	>256
Rutin	>256	>256	>256	>256	>256	>256	>256
p-Coumaric acid	>256	>256	>256	>256	>256	>256	>256
Oxacillin	512	128	256	256	512	8	0.13
Ciprofloxacin	8	1	16	8	<0.13	0.25	<0.13
Norfloxacin	128	8	64	64	1	2	0.5
Erythromycin	>1024	>128	>1024	>128	0.13	>1024	2
Tetracycline	4	0.25	128	64	128	0.13	0.13
Vancomycin	0.25	0.25	0.25	0.25	0.25	0.25	<0.25

Table 2. Antibacterial Activity of C2 and C3

Compound	MIC (μg/ml)				
	E. faecium FN-1	*E. faecalis* NCTC12201	*S. pneumoniae* R6	*P. aeruginosa* PAO1	*S. marcescens* NUSM8906
C2	8	4	>16	>16	>16
C3	8	4	>16	>16	>16
Vancomycin	>128	>128	<0.25	>128	>128
Kaempferol	>256	>256	256	>256	>256

Thus, each one of these compounds alone is not enough for the inhibition of MRSA growth. Compounds that possess structures similar to C2 and C3, *i.e.*, quercetin, quercitrin (quercetin 3-rhamnoside), isoquercetin (quercetin 3β-D-glucoside) and rutin (quercetin 3-rhamnoglucoside) (Fig. 1), showed no detectable anti-MRSA and anti-MSSA activities (Table 1). Thus, it seems at the present time that the intact structures of C2 and C3 are important for antibacterial activity.

Antibacterial Activity on Some Other Bacteria We also tested the antibacterial activities of C2 and C3 on some other bacteria such as *E. faecium* FN-1, *E. faecalis* NCTC12210, *Streptococcus pneumoniae* R6, *Pseudomonas aeruginosa* PAO1 and *Serratia marcescens* NUSM8906. *E. faecium*, *E. faecalis* and *S. pneumoniae* are Gram-positive bacteria, and *P. aeruginosa* and *S. marcescens* are Gram-negative bacteria. We observed MIC values being 8 and 4 μg/ml for *E. faecium* FN-1 and *E. faecalis* NCTC12201, respectively, with both C2 and C3 (Table 2). Since *E. faecium* FN-1 and *E. faecalis* NCTC12201 are VRE strains, the MIC of vancomycin with these strains was very high, higher than 128 μg/ml (Table 2). Thus, C2 and C3 are moderately effective on the VRE strains tested. On the other hand, the MIC value of C2 and C3 were higher than 16 μg/ml for *S. pneumoniae*, *P. aeruginosa* and *S. marcescens*. Kaempferol was not effective on all of these bacteria (Table 2).

DISCUSSION

We isolated anti-MRSA compounds from *L. nobilis* (laurel) leaves and identified them as C2 and C3 (Fig. 1). Laurel is an evergreen tree widespread in the Mediterranean area and Europe, and is used as a folk medicine. Decoction or tea of leaves is often used as a carminative, an intestinal and gastric antispasmodic, an anti-diarrheal agent, for treating rheumatic pain, in treating diseases of the respiratory tract, as a cough sedative, and treating asthma and cardiac diseases.[14] This means that the laurel contains many medicinal compounds. It seems that C2 and C3 are two such medicinal compounds in laurel. The C2 and C3 compounds belong to the flavonoids. Many research groups have so far isolated and determined the structures of flavonoids that possess antibacterial activity, or quantified the activity of commercially available flavonoids.[15] The MIC values of C2 and C3 with MRSA are at the level of μg/ml. These values are in the lowest MIC value range against bacteria among flavonoids. In other words, C2 and C3 possess very strong antibacterial activity among the flavonoids. The anti-MRSA activity of C2 and C3 was much higher than those of chemotherapeutics such as oxacillin, ciprofloxacin, norfloxacin, erythromycin and tetracycline (Table 1). The activity was a little weaker than that of vancomycin, the clinical first-choice drug for MRSA infections. It should be pointed out that the C2 and C3 compounds are moderately effective on VRE (Table 2), of which vancomycin is ineffective. Thus, C2 and C3 could be seeds for anti-VRE agents.

The structure–activity relationship for the flavanones, depicted from their MIC measurement, has been reported with MRSA.[16] The study indicated that 5,7-dihydroxylation of the A ring and 2′,4′- or 2′,6′-dihydroxylation of the B ring in the flavanone structure are important for significant anti-MRSA activity. The C2 and C3 carry 4′-hydroxyl substitution of the A ring, 5,7-dihydroxyl substitutions in the B ring (Fig. 1). Thus, structures of C2 and C3 mostly fit this structure–activity relationship. Furthermore, it has been reported that substitution with 3-*O*-acyl chains with C_8 and C_{10} in the C ring also enhanced the anti-staphylococcal activity of flavonoids belonging to the flavan-3-ol class.[17] The C2 and C3 compounds possess 3-*O*-acylated rhamnose. The acyl (coumaroyl) group in C2 and C3 contains 10 carbon atoms. Thus, this portion would also contribute to the strong anti-MRSA activity of C2 and C3. The C2 and C3 compounds showed almost the same MICs with several MRSA strains and an MSSA strain (Table 1). The C2 and C3 compounds are isomers regarding the acyl (coumaroyl) portion. This result suggests that the isomeric fine structure of this portion is not important for antibacterial activity of C2 and C3. We propose that the two acyl groups in C2 and C3 are important for the compounds to enter into MRSA cells. Similarly, 3-*O*-acyl chains with C_8 and C_{10} would be important for flavan-3-ols entry into cells.

It has been reported that laurel leaves containing glycosylated flavonoids and C2 have been identified in *Laurus nobilis*,[18] *Ocotea vellosiana* (Lauraceae)[19] and in *Platanus acenifolia* (Platanaceae).[20] Bloor isolated C2 from *Pentachondra pumila* (Epacridaceae) and reported an anti-MRSA effect.[21] However, he has not reported MIC of the compound with MRSA. Thus, it was not known how active the C2 was against MRSA. Recently, C3 has been identified in *Cinnamomum kotoense* (Lauraceae) and has been shown to possess an anti-inflammatory effect.[22]

So far, antibacterial mechanisms of various flavonoids have been investigated. The best-known antibacterial mechanisms of the flavonoids include inhibition of nucleic acid synthesis, inhibition of cytoplasmic membrane function, and inhibition of energy metabolism.[15] Glycosylated flavonoids have been reported to show effects as inhibitors of bacterial type II topoisomerases, such as DNA gyrase and topoisomerase IV.[23] Ohemeng *et al.* reported inhibitory activity of 14 flavonoids including kaempferol against *E. coli* DNA

gyrase.[24] However, kaempferol itself did not show anti-MRSA activity (Table 1). Thus, it is likely that after C2 and C3 get into *S. aureus* cells and that these compounds, or their cleavage product kaempferol, or kaempferol 3-*O*-rhamnoside, inhibit DNA gyrase and topoisomerase IV.

Acknowledgments We thank Dr. Manuel Varela of Eastern New Mexico University for critically reading the manuscript. This research was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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