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Flavanones with Potent Antibacterial Activity against Methicillin-resistant *Staphylococcus aureus*

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Abstract—With the therapeutic concept of using the defensive ability of plants against microbial infections, phytoalexin, an antimicrobial phytochemical was studied for its ability to inhibit the growth of methicillin-resistant *Staphylococcus aureus* (MRSA). Extracts from *Sophora exigua* (Leguminosae) were fractionated by serial chromatography and the anti-MRSA activity of each fraction was determined by the agar-plate method. Among the active isolates, 5,7,2′,6′-tetrahydroxy-6-isoprenyl-8-lavandulyl-4′-methoxyflavanone (exiguaflavanone D) completely inhibited the growth of all the MRSA strains examined at the concentration of 1·56–6·25 $\mu g\,mL^{-1}$, and 5, 2′,6′-trihydroxy-8-lavandulyl-7-methoxyflavanone (exiguaflavanone B) inhibited at a concentration of 50 $\mu g\,mL^{-1}$. This former compound is expected to be a phytotherapeutic agent for MRSA infections as an alternative to conventional antibiotics with unwanted side-effects or the appearance of antibiotic-resistant bacteria.

Much attention has recently been paid to methicillin-resistant *Staphylococcus aureus* (MRSA) which is responsible for worldwide outbreaks of nosocominal infections (Eykyn 1988; Marples & Cooke 1988; Maple et al 1989). In Japan, the incidence of MRSA infections in hospitals has dramatically increased since the early 1980s, and MRSA infections have become a critical problem as the major cause of mortality among hospitalized patients (Thompson et al 1982; Townsend et al 1987). Antibiotics are usually used for treating MRSA infections, but their use evokes inevitable problems with the appearance of side-effects and eventually resistant bacteria.

The present study was performed to find anti-MRSA activity in plants. Plants synthesize various antimicrobial phytochemicals as a self-defense system against microbial infections. Phytoalexins are those compounds with broad antimicrobial spectra which are biosynthesized in response to infectious stimuli (Dixon et al 1983). Many phytoalexins are synthesized by various edible plants, and are different in chemical structure from known antibiotics (Kurosaki & Nishi 1983; Ebel & Grisebach 1988). In addition to a reliable antibacterial effect derived from their natural functions, we would expect these natural products to have fewer side-effects and less tendency to acquire resistance compared with conventional semi-synthetic antibiotics.

Materials and Methods

Fractionation of phytochemicals

The dried roots (120 g) of *Sophora exigua*, collected in Thailand, were pulverized and extracted with acetone (500 mL × 2). Chromatographic fractionation of phytochemicals was performed according to methods reported previously (Ruangrungsi et al 1992; Iinuma et al 1993). In brief, the combined extracts were fractionated on a 5·0 × 50 cm column packed with Kiesel gel 60 (70–230 mesh; Merck, Darmstadt, Germany) eluted with *n*-hexane and acetone (5 : 1 to 1 : 1, v/v) at 30 mL min^{-1}. Four fractions (200 mL) were collected with monitoring by thin layer chromatography (TLC) using Kiesel gel F254 TLC (0·25 mm; Merck) and chloroform : methanol (10 : 1), and antibacterial activity.

The fourth fraction was further fractionated on the same column with an eluent of chloroform : methanol (20 : 1 to 5 : 1) and an elution rate of 15 mL min^{-1}. Six fractions (500 mL) were collected with monitoring by TLC and antibacterial activity.

The first fraction of this second separation was further fractionated on a 3·0 × 50 cm Kiesel gel 60 column with an eluent of *n*-hexane : acetone (10 : 1) at 10 mL min^{-1}, to give compounds 1, 2, and 3. After confirming their purity by TLC, the three isolates were subjected to the determination of minimum inhibitory concentration (MIC) and structural identification.

Preparation of bacteria

The MRSA and methicillin-sensitive *Staphylococcus aureus* (MSSA) strains listed in Table 1 were kindly supplied by Dr S. Asai and from laboratory stock cultures, respectively. The clinical isolates were defined as MRSA based on their resistance to methicillin and oxacillin, according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS 1990). After culturing all strains on Mueller-Hinton agar (Difco, Detroit, MI), the cells were resuspended in Mueller-Hinton broth (Difco) to give 10^8 colony forming units mL^{-1}, and then inoculated.

Determination of anti-MRSA activity

After evaporating all fractions to dryness, the residues were dissolved in triethanolamine : water (30 : 70) to give concentrations of 12·5–250 $\mu g\,mL^{-1}$ in the medium, and added to Mueller-Hinton agar media. This level of tri-

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Table 1. Minimum inhibitory concentrations of antibiotics.

Bacteria	Minimum inhibitory concn ($\mu g\,mL^{-1}$)			
	Methicillin	Oxacillin	Gentamicin	Vancomycin
Methicillin-resistant *Staphylococcus aureus*				
S. aureus (G 112)	>100	>100	>100	<1·56
S. aureus (H 103)	>100	>100	>100	1·56
S. aureus (G 31)	>100	>100	100	<1·56
S. aureus (G 113)	>100	>100	100	1·56
S. aureus (G 114)	>100	>100	100	1·56
S. aureus (S 464)	>100	>100	100	1·56
S. aureus (S 239)	>100	>100	50·0	<1·56
S. aureus (G 86)	>100	>100	25·0	<1·56
S. aureus (H 310)	>100	>100	25·0	1·56
S. aureus (G 47)	>100	>100	<1·56	<1·56
S. aureus (G 91)	100	25·0	1·56	1·56
S. aureus (G 74)	12·5	12·5	>100	<1·56
Methicillin-sensitive *Staphylococcus aureus*				
S. aureus (ATCC 25923)	1·56	<1·56	<1·56	1·56
S. aureus (ATCC 29213)	1·56	<1·56	<1·56	<1·56
S. aureus (Tazaki)	1·56	<1·56	<1·56	<1·56
S. aureus (209 P)	1·56	<1·56	<1·56	<1·56

ethanolamine (0·6%) added to the control showed no influence on the growth of any bacteria. The inoculated plates were aerobically incubated at 37°C. The antibacterial activity was defined as + when no colony was observed after incubation for 48 h.

The MIC values of the three isolated compounds were determined by a twofold serial agar dilution method. MIC values were defined as the lowest concentrations at which no colony was observed after incubation for 48 h. The MIC values of methicillin, oxacillin, gentamicin, and vancomycin (Sigma, St Louis, MO) were similarly determined.

Structural identification of active isolates

The isolated compounds were subjected to elemental analysis and spectral measurements including HR-MS, EI-MS, ^{1}HNMR, ^{13}CNMR, and UV/vis in a manner similar to previous reports (Ruangrungsi et al 1992; Iinuma et al 1993). Their chemical structures were identified based on spectral data.

Results

Susceptibility to antibiotics

The MIC values of the staphylococcal strains to antibiotics are shown in Table 1. Except for *S. aureus* (G 74), most of the MRSA clinical isolates were highly resistant, not only to methicillin but also oxacillin, whereas the MSSA strains were susceptible to all antibiotics. The growth of all the MRSA strains was uniformly inhibited by vancomycin at 1·56 $\mu g\,mL^{-1}$ or less.

Anti-MRSA activity

The anti-MRSA activity against staphylococcal growth at 250 $\mu g\,mL^{-1}$ was confined to the fourth fraction which, however, showed no antibacterial activity to bacteria other than *S. aureus*. In the second fractionation, only the first fraction inhibited the growth of both MRSA and MSSA strains at the concentration of 25–50 $\mu g\,mL^{-1}$.

MIC values of isolated compounds

The MIC values of compound 1 and compound 2 are shown in Table 2. Among the three isolates, compound 1 and compound 2 showed anti-MRSA activity; compound 3 failed to inhibit bacterial growth at 50 $\mu g\,mL^{-1}$. The MIC values of the most active compound 1 ranged from 1·56 to 6·25 $\mu g\,mL^{-1}$ for both MRSA and MSSA. Compound 2 was less active than compound 1.

Structural identification of compound 1 and compound 2

Compound 1, obtained as a pale yellow solid, showed a molecular ion at m/z 522 in HR-MS which corresponded to $C_{31}H_{38}O_7$. From spectral data, it was identified as 5,7,2′,6′-tetrahydroxy-6-isoprenyl-8-lavandulyl-4′-methoxyflavanone, exiguaflavanone D (Iinuma et al 1993) (Fig. 1).

Compound 2, obtained as a pale yellow viscous oil, showed a molecular ion at m/z 438 in HR-MS which corresponded to $C_{26}H_{30}O_6$. Its spectral data agreed with those of 5,2′,6′-trihydroxy-8-lavandulyl-7-methoxyflavanone, exiguaflavanone B (Ruangrungsi et al 1992) (Fig. 1).

Table 2. Minimum inhibitory concentrations of the phytochemicals isolated as compound 1 and compound 2.

Bacteria	Minimum inhibitory concn ($\mu g\,mL^{-1}$)	
	Compound 1	Compound 2
Methicillin-resistant *Staphylococcus aureus*		
S. aureus (G 112)	6·25	50·0
S. aureus (H 103)	6·25	50·0
S. aureus (G 31)	6·25	50·0
S. aureus (G 113)	6·25	50·0
S. aureus (G 114)	6·25	50·0
S. aureus (S 464)	3·13	50·0
S. aureus (S 239)	6·25	50·0
S. aureus (G 86)	6·25	50·0
S. aureus (H 310)	3·13	50·0
S. aureus (G 47)	3·13	50·0
S. aureus (G 91)	6·25	50·0
S. aureus (G 74)	3·13	50·0
Methicillin-sensitive *Staphylococcus aureus*		
S. aureus (ATCC 25923)	3·13	50·0
S. aureus (ATCC 29213)	6·25	50·0
S. aureus (Tazaki)	1·56	50·0
S. aureus (209 P)	3·13	50·0

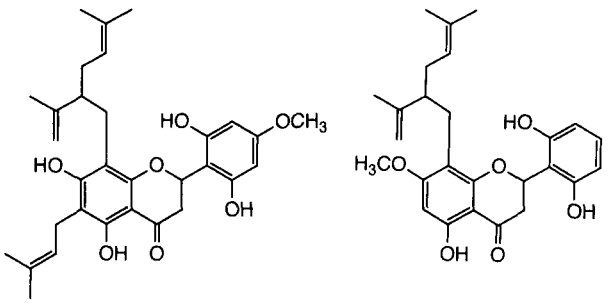

FIG. 1. Chemical structures of the anti-MRSA flavanones isolated as compound 1 and compound 2.

Discussion

Vancomycin and arbekacin with intensive anti-MRSA activity have been used as first-choice agents for severe MRSA infections (Kobayashi 1992). In addition to side-effects, however, new organisms can develop which are resistant to such antibiotics (Schwalbe et al 1987; Sowa et al 1991). Although teicoplanin (Gorzynski et al 1989), daptomycin (Hodinka et al 1987), RP 59500 (Fass 1991), SK&F 104662 (Yao et al 1989), and an anti-MRSA dipeptide (Funabashi et al 1993) have been developed one after another, they also are expected to suffer from the same problems because they are based on the conventional concept of antibiotic therapy.

Plants are important sources for various pharmaceutical agents and useful pharmacological activity has been widely exploited in phytochemicals (Balandrin et al 1985; D'Ocón et al 1992). Catechins isolated from tea leaves possess anti-MRSA activity, although not as intensive as antibiotics (Toda et al 1991). Essential oils from plants show MIC values of 175 $\mu g\,mL^{-1}$ to *Staphylococcus aureus* (Cruz et al 1993). Protoanemonin from *Ranunculus bulbosus* inhibits the growth of MRSA and MSSA at the concentration range of 31·25–62·5 $\mu g\,mL^{-1}$ (Didry et al 1993). Chalcones (Szajda & Kedzia 1991), xanthoangelol and 4-hydroxyderricin from *Angelica keiskei* Koidzumi (Inamori et al 1991), and isoflavanones from *Erythrina* × *bidwilli* (Iinuma et al 1992) inhibit staphylococcal growth. However, the MIC values of such phytochemicals range only from 50 to 500 $\mu g\,mL^{-1}$ or more, except for xanthoangelol and 4-hydroxyderricin with the MIC values of 3.12–6.25 $\mu g\,mL^{-1}$ to two MSSA strains.

Among antimicrobial phytochemicals, phytoalexins have a unique character. They are low-molecular weight compounds which are biosynthesized by plants in response to microbial infections (Dixon et al 1983). They are concentrated at infected sites and participate in the plant's self-defense system against pathogenic microorganisms. One of the phytoalexins, 6-methoxymellein, derived from carrots, inhibits the growth of *Staphylococcus aureus* (Kurosaki & Nishi 1983).

In the present study, serial chromatographic fractionations and screening for anti-MRSA activity have revealed that two flavanone phytoalexins derived from *S. exigua* possess anti-MRSA activity. The more active 5,7,2′,6′-tetrahydroxy-6-isoprenyl-8-lavandulyl-4′-methoxyflavanone inhibited the growth of both MRSA and MSSA at the concentration of 1·56–6·25 $\mu g\,mL^{-1}$, and its antibacterial spectrum is relatively specific to *Staphylococci*. Vancomycin inhibited the growth of MRSA at 1·56 $\mu g\,mL^{-1}$ or less, but the MIC values of other antibiotics varied depending on the degree of resistance. On the other hand, the flavanone phytoalexin was uniformly active against all strains of MRSA and MSSA, although its inhibitory concentrations were slightly higher than those of vancomycin.

In a preliminary experiment to ascertain structure-activity relationships, the anti-MRSA activity was determined in various flavonoid phytoalexins with different structures. However, an anti-MRSA activity comparable with that of 5,7,2′,6′-tetrahydroxy-6-isoprenyl-8-lavandulyl-4′-methoxyflavanone was not obtained from any phytochemical flavonoids tested. When the C ring of the pilot flavanones was opened to specify the active site, the anti-MRSA activity was completely lost. It is presumed that the skeleton of flavanone is essential and that substitution of the A ring with hydroxyl and certain alkyl groups is additionally needed to elevate the anti-MRSA activity.

The present anti-MRSA compound is a flavonoid, as are nearly half of phytoalexins which have been structurally characterized (Harborne 1988). Reduced toxicity and side-effects may be expected as phytochemical flavonoids widely occur in edible plants and have been used in medicine (Havsteen 1983; Cody 1988).

In addition to MRSA, oxacillin-resistant *Staphylococcus aureus* (ORSA) has become another cause of hospital- and community-acquired infections (Brumfitt & Hamilton-Miller 1989). Although quinolone antibiotics were shown to be effective against ORSA (Aldridge et al 1985; Smith & Eng 1985), quinolone-resistant strains of ORSA have been frequently isolated (Kayser & Novak 1987; Daum et al 1990; Aldridge et al 1992). At least in the in-vitro experiment, the present flavanone phytoalexin shows intensive antibacterial activity against both MRSA and ORSA. It is also promising as a phytotherapeutic agent against ORSA infections.

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References

Aldridge, K. E., Janney, A., Sanders, C. V. (1985) Comparison of the activities of coumermycin, ciprofloxacin, teicoplanin, and other non-β-lactam antibiotics against clinical isolates of methicillin-resistant *Staphylococcus aureus* from various geographical locations. Antimicrob. Agents Chemother. 28: 634–638

Aldridge, K. E., Jones, R. N., Barry, A. L., Gelfand, M. S. (1992) In vitro activity of various antimicrobial agents against *Staphylococcus aureus* isolates including fluoroquinolone- and oxacillin-resistant strains. Diagn. Microbiol. Infect. Dis. 15: 517–521

Balandrin, M. F., Klocke, J. A., Wurtele, E. S., Bollinger, W. H. (1985) Natural plant chemicals: sources of industrial and medicinal materials. Science 228: 1154–1160

Brumfitt, W., Hamilton-Miller, J. (1989) Methicillin-resistant *Staphylococcus aureus*. N. Engl. J. Med. 320: 1188–1196

Cody, V. (1988) Crystal and molecular structures of flavonoids. In: Cody, V., Middleton, E., Jr., Harborne, J. B., Beretz, A. (eds) Plant Flavonoids in Biology and Medicine II. Biochemical,

Cellular, and Medicinal Properties. Alan R. Liss, New York, pp 29–44

Cruz, T., Cabo, M. M., Castillo, M. J., Jimenez, J., Ruiz, C., Ramos-Cormenzana, A. (1993) Chemical composition and antimicrobial activity of the essential oils of different samples of *Thymus baeticus* Boiss. Phytother. Res. 7: 92–94

Daum, T.E., Schaberg, D.R., Terpenning, M.S., Sottile, W.S., Kauffman, C.A. (1990) Increasing resistance of *Staphylococcus aureus* to ciprofloxacin. Antimicrob. Agents Chemother. 34: 1862–1863

Didry, N., Dubreuil, L., Pinkas, M. (1993) Microbiological properties of protoanemonin isolated from *Ranunculus bulbosus*. Phytother. Res. 7: 21–24

Dixon, R. A., Dey, P. M., Lamb, C. J. (1983) Phytoalexins: enzymology and molecular biology. Adv. Enzymol. 55: 1–69

D'Ocón, P., Blázquez, M. A., Bermejo, A., Anselmi, E. (1992) Tetrandrine and isotetrandrine, two bisbenzyltetrahydroisoquinoline alkaloids from *Menispermaceae*, with rat uterine smooth muscle relaxant activity. J. Pharm. Pharmacol. 44: 579–582

Ebel, J., Grisebach, H. (1988) Defense strategies of soybean against the fungus *Phytophthora megasperma* f.sp. *glycinea*: a molecular analysis. Trends Biochem. Sci. 13: 23–27

Eykyn, S. J. (1988) Staphylococcal sepsis. The changing pattern of disease and therapy. Lancet i: 100–104

Fass, R. J. (1991) In vitro activity of RP 59500, a semisynthetic injectable pristinamycin, against staphylococci, streptococci, and enterococci. Antimicrob. Agents Chemother. 35: 553–559

Funabashi, Y., Tsubotani, S., Koyama, K., Katayama, N., Harada, S. (1993) A new anti-MRSA dipeptide, TAN-1057 A. Tetrahedron 49: 13–28

Gorzynski, E. A., Amsterdam, D., Beam, T. R., Jr., Rotstein, C. (1989) Comparative in vitro activities of teicoplanin, vancomycin, oxacillin, and other antimicrobial agents against bacteremic isolates of Gram-positive cocci. Antimicrob. Agents Chemother. 33: 2019–2022

Harborne, J. B. (1988) Flavonoids in the environment: structure-activity relationships. In: Cody, V., Middleton, E., Jr., Harborne, J. B., Beretz, A. (eds) Plant Flavonoids in Biology and Medicine II. Biochemical, Cellular, and Medicinal Properties. Alan R. Liss, New York, pp 17–27

Havsteen, B. (1983) Flavonoids, a class of natural products of high pharmacological potency. Biochem. Pharmacol. 32: 1141–1148

Hodinka, R. L., Jack-Wait, K., Wannamaker, N., Walden, T. P., Gilligan, P. H. (1987) Comparative in vitro activity of LY 146032 (daptomycin), a new lipopeptide antimicrobial. Eur. J. Clin. Microbiol. 6: 100–103

Iinuma, M., Tanaka, T., Mizuno, M., Yamamoto, H., Kobayashi, Y., Yonemori, S. (1992) Phenolic constituents in *Erythrina* × *bidwilli* and their activity against oral microbial organisms. Chem. Pharm. Bull. 40: 2749–2752

Iinuma, M., Yokoyama, J., Ohyama, M., Tanaka, T., Mizuno, M., Ruangrungsi, N. (1993) Seven phenolic compounds in the roots of *Sophora exigua*. Phytochemistry 33: 203–208

Inamori, Y., Baba, K., Tsujibo, H., Taniguchi, M., Nakata, K., Kozawa, M. (1991) Antibacterial activity of two chalcones, xanthoangelol and 4-hydroxyderricin, isolated from the root of *Angelica keiskei* Koidzumi. Chem. Pharm. Bull. 39: 1604–1605

Kayser, F. H., Novak, J. (1987) In vitro activity of ciprofloxacin against Gram-positive bacteria: an overview. Am. J. Med. 82 (Suppl. 4A): 33–39

Kobayashi, Y. (1992) Vancomycin and arbekacin, drugs of treatment for MRSA infections. Nippon Rinsho 50: 1054–1059 (in Japanese)

Kurosaki, F., Nishi, A. (1983) Isolation and antimicrobial activity of the phytoalexin 6-methoxymellein from cultured carrot cells. Phytochemistry 22: 669–672

Maple, P. A., Hamilton-Miller, J. M., Brumfitt, W. (1989) World-wide antibiotic resistance in methicillin-resistant *Staphylococcus aureus*. Lancet i: 537–540

Marples, R. R., Cooke, E. M. (1988) Current problems with methicillin-resistant *Staphylococcus aureus*. J. Hosp. Infect. 11: 381–392

National Committee for Clinical Laboratory Standards (1990) Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 2nd edn. Approved standard. NCCLS document M7-A2. National Committee for Clinical Laboratory Standard, Villanova, PA

Ruangrungsi, N., Iinuma, M., Tanaka, T., Ohyama, M., Yokoyama, J., Mizuno, M. (1992) Three flavanones with a lavandulyl group in the roots of *Sophora exigua*. Phytochemistry 31: 999–1001

Schwalbe, R. S., Stapleton, J. T., Gilligan, P. H. (1987) Emergence of vancomycin resistance in coagulase-negative staphylococci. N. Engl. J. Med. 316: 927–931

Smith, S. M., Eng, R. H. K. (1985) Activity of ciprofloxacin against *Staphylococcus aureus*. Antimicrob. Agents Chemother. 27: 688–691

Sowa, S., Masumi, N., Inouye, Y., Nakamura, S., Takesue, Y., Yokoyama, T. (1991) Susceptibility of methicillin-resistant *Staphylococcus aureus* clinical isolates to various antimicrobial agents. Hiroshima J. Med. Sci. 40: 137–144

Szajda, M., Kedzia, B. (1991) New *N*-substituted derivatives of 3-azachalcone of potential antimicrobial activity. Pharmazie 46: 745–746

Thompson, R. L., Cabezudo, I., Wenzel, R. P. (1982) Epidemiology of noscomial infections caused by methicillin-resistant *Staphylococcus aureus*. Ann. Intern. Med. 97: 309–317

Toda, M., Okubo, S., Hara, Y., Shimamura, T. (1991) Antibacterial and bactericidal activities of tea extracts and catechins against methicillin resistant *Staphylococcus aureus*. Jpn. J. Bacteriol. 46: 839–845 (in Japanese)

Townsend, D. E., Ashdown, N., Bolton, S., Bradley, J., Duckworth, G., Moorhouse, E. C., Grubb, W. B. (1987) The international spread of methicillin-resistant *Staphylococcus aureus*. J. Hosp. Infect. 9: 60–71

Yao, J. D., Eliopoulos, G. M., Moellering, R. C., Jr. (1989) In vitro activity of SK&F 104662, a new glycopeptide antibiotic. Antimicrob. Agents Chemother. 33: 965–967