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Article

Antibacterial and Synergistic Activity of Prenylated Chalcone Isolated from the Roots of *Sophora flavescens*

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7,9,2',4'-Tetrahydroxy-8-isopentenyl-5-methoxychalcone (THIPMC), isolated from the roots of *Sophora flavescens* Ait., was found to be active against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE), either alone or in combination with ampicillin (AM) or gentamicin (GM), *vis* checkerboard assay. Minimum inhibitory concentrations ranged from 1 to 8 μg/mL for THIPMC, from 128 to 1024 μg/mL for AM, and from 128 to 512 μg/mL for GM, respectively. The combinations of THIPMC plus AM or GM yielded a fractional inhibitory concentration index ranging from 0.188 to 0.375 μg/mL, thereby indicating a synergistic effect. These findings suggest that THIPMC alone or in combination with antibiotics against MRSA might be useful for controlling MRSA infections. However, VRE infection was only effectively treated by THIPMC alone.

Key words: enterotoxin gene, MRSA, *Sophora flavescens,* THIPMC, VRE

Sophorae radix, the dried roots of *Sophorae flavescens* A_{ITON} (Leguminosae), have been shown to possess a variety of pharmacological properties, including antibacterial [Kuroyanagi *et al*., 1999], antimalarial [Kim *et al*., 2004], antiprotozoal [Youn *et al*., 2004], anti-pyretic and anti-inflammatory [Hsiang *et al*., 2001] properties, and to contain more than 50 phytochemicals, which include quinolizidine alkaloids, triterpenoid saponins, and prenylated flavonoids [Kim *et al*., 2004; Botta *et al*., 2005]. Recent pharmaceutical studies have shown that the lavandulyl side chain is essential for the antitumor activities and phospholipase-Cγl-inhibitory activities of flavonoids isolated from this plant [DeNaeyer *et al*., 2004].

Gram-positive bacteria are common causes of nosocomial infections. Since outbreaks of vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) were first reported, outbreaks of nosocomial VRE and MRSA infection have been increasingly reported worldwide. MRSA and/or VRE are representative of the drug resistant bacteria responsible for these infectious diseases [Rice, 2006], and the causes of this rising incidence include the inappropriate and excessive use of antibiotics and insufficient hygiene in the hospital environment [Fujita, 2005; Rice, 2006]. Multi-drug resistant and infectious diseases caused by MRSA present intensely difficult problems [Linton *et al*., 1988]. Thus, we have undertaken a work program to discover effective growth inhibitors of MRSA and VRE

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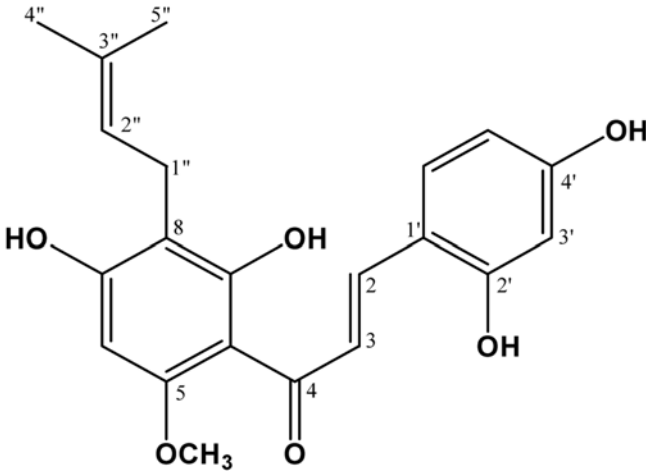

Fig. 1. The chemical structure of 7,9,2',4'-tetrahydroxy-8-isopentenyl-5-methoxychalcone (THIPMC).

[Jeong *et al*., 2006; Choi *et al*., 2009]. Sophorae radix, the dried roots of *Sophora flavescens*, has been used in Korean medicine for treatment of diarrhea, inflammation, abscess, dysentery [Kuroyanagi *et al*., 1999; Hsiang *et al*., 2001]. In the course of our ongoing project on the detection of bioactive compounds from medicinal plants, the $CHCl_3$ fraction of *S. flavescens* was found to have the compound that have an antibacterial activity (7,9,2',4'-tetrahydroxy-8-isopentenyl-5-methoxychalcone (THIPMC, Fig. 1). In the present study, we investigated the antimicrobial activity of THIPMC and synergistic effect of the mixture of ampicillin, cephalothin, clindamycin or penicillin, with THIPMC against MRSA and VRE.

Materials and Methods

Plant materials. The roots of *S. flvescens* used in the present study were collected during October 2003 from Jinan, Jeonbuk Province, South Korea. The plant was identified and authenticated by Prof. Hong Jun Kim at the College of Oriental Medicine, Woosuk University. A voucher specimen (No. JSI0903) has been deposited at the department Oriental Medicine, Woosuk University, Republic of Korea.

Instruments. The NMR spectrum of THIPMC was recorded in CD_3OD on JEOLJMN-EX 400 MHz (JEOL, Tokyo, Japan) and Bruker DMX-600 MHz spectrometers (Bruker, Karlsruhe, Germany). ^{1}H-^{1}H COSY, HMQC, and HMBC experiments were recorded with gradient enhancements using shaped gradient pluses. EI/MS (70 eV) was performed on a VG-VSEQ mass spectrometer (VG Analytical, U.K); optical rotation was obtained using an ADP220 digital polarimeter (Bellingham & Stanley Co., Ltd, Paris); and preparative HPLC was carried out using a JAIGEL GS column (Japan Analytical Industry Co., Ltd, Tokyo).

Preparation of bacterial strains. MRSA and VRE strains were clinical isolates from Wonkwang University Hospital (Iksan, Korea). The MRSA strains were defined on the basis of the occurrence of the *mecA* gene and of their resistance to ampicillin and oxacillin, VRE strains were defin on the basis of expression of the *vanA* gene and of their resistance to vancomycin according to the guidelines of the Clinical and Laboratory Standards Institute. The MRSA and VRE strains were defined after culturing all strains on Mueller-Hinton agar (MHA; Difco, Detroit, MI), all bacteria were resuspended in Mueller-Hinton broth (MHB; Difco, Detroit, MI) to give 1×10^8 CFU (colony-forming units)/mL; the resuspended bacteria were then incubated.

Extraction and isolation of the active compound. The shade-dried roots of *S. flavescens* (3.2 kg) were crushed and extracted three times with MeOH under reflux. The MeOH extract so obtained was concentrated, suspended in H_2O, and sequentially partitioned with $CHCl_3$, EtOAc, and *n*-BuOH. The $CHCl_3$ fraction (120 g) was then subjected to silica gel column chromatography using $CHCl_3$-EtOAc-MeOH (15:1:1) as eluant to afford nine fractions. Biological activity was found to be concentrated in fraction 4 (14 g), which was further purified by silica gel column chromatography using $CHCl_3$-EtOAc-MeOH (10:1:1) as eluant to yield four subfractions (fraction 1-4). Fraction 2 (2.7 g) exhibited MIC values of 32 μg/mL against *S. aureus* ATCC 33592, and was further fractionated using Sephadax LH-20 and $CHCl_3$-EtOAc-MeOH (10:1:1), and fraction 2 of this fractionation was purified by preparative HPLC (JALGS column, MeOH at 3 mL/min and at 210 nm UV) to yield compound.

7,9,2',4'-tetrahydroxy-8-isopentenyl-5-methoxychalcone (THIPMC). a pale yellow amorphous powder, $[\alpha]_D^{25}$ + 2.6 (*c*0.01, MeOH); EI/MS *m/z* 370 $[M]^+$, ^{1}H-NMR (500 MHz, Acetone-d_6) δ 14.86 (1H, s, 9-OH), 8.15 (1H, d, *J*= 15.5 Hz, H-2), 7.99 (1H, d, *J*=15.5 Hz, H-3), 7.52 (1H, d, *J*=8.5 Hz, H-6'), 6.50 (1H, d, *J*=2.0 Hz, H-3'), 6.44 (1H, dd, *J*=8.5, 2.0 Hz, H-5'), 6.13 (1H, s, H-6), 5.24 (1H, m, H-2''), 3.89 (3H, s, C_5-O$\underline{CH_3}$), 3.27 (2H, d, *J*=7.5Hz, H-1''), 1.75 (3H, s, H-5''), 1.63 (3H, s, H-4''). ^{13}C-NMR (100 MHz, Acetone-d_6) δ 139.3 (C-2), 124.3 (C-3), 193.8 (C-4), 166.5 (C-5), 91.8 (C-6), 162.6 (C-7), 109.3 (C-8), 162.0 (C-9), 106.6 (C-10), 116.0 (C-1'), 159.7 (C-2'), 103.8 (C-3'), 161.9 (C-4'), 109.0 (C-5'), 131.3 (C-6'), 22.4 (C-1''), 125.0 (C-2''), 131.0 (C-3''), 26.2 (C-4''), 18.2 (C-5''), 56.3 (C_5-O$\underline{C}H_3$).

Determination of minimum inhibitory concentrations (MICs). The MIC was performed by the microdilution

broth method. Serial two-fold dilutions of ampicillin, gentamicin, THIPMC, vancomycin was prepared in sterile 96-well micro plates and microtube with concentrations ranging MIC by using MHB. The MRSA and VRE suspensions were adjusted to the 0.5 McFarland standards (approximately 1×10^8 CFU/mL). Final inoculums were adjusted to the 1×10^6 CFU/mL. The MHB was supplemented with serial antibiotics concentrations ranging from 0.25 to 1024 μg/mL, and with THIPMC at concentrations from 0.24 to 1,000 μg/mL. The data were reported as MICs, the lowest concentration of antibiotics inhibiting visible growth after 16 hr of incubation at 37°C [Shahverdi *et al*., 2007].

Kinetics of bacterial killing. To study the combined effects of time and antimicrobial agent concentration on bacterial growth, the kinetics of bacterial killing curves

Table 1. MIC characteristics of THIPMC against MRSA and VRE isolates obtained from Wonkwang University Hospital

Strain		MIC (μg/mL)
MRSA	*S. aureus* (33592)	0.97
	CL2	3.90
	CL3	0.97
	CL6	3.90
	CL9	15.6
	CL19	1.95
VRE	VRE24	15.6
	CL186	7.8
	CL189	15.6
	CL232	7.8
	CL233	15.6

Table 2. Synergic effect of THIPMC, AM, and GM against MRSA

Strains	Agent	MIC [b] (μg/mL)		FIC (μg/mL)	FICI[d]	Outcome
		Alone	Combination[c]			
S. aureus (33592)	Ampicillin	256	2.00	0.00	1.00	additive
	[a]THIPMC	0.97	0.97	1.00		
	Gentamicin	512	0.50	0.00	0.25	Synergistic
	THIPMC	0.97	0.24	0.25		
CL2	Ampicillin	256	8.00	0.00	0.5	Synergistic
	THIPMC	3.90	1.95	0.5		
	Gentamicin	256	128	0.00	0.5	Synergistic
	THIPMC	3.90	1.95	0.5		
CL3	Ampicillin	256	2.00	0.00	1.0	additive
	THIPMC	0.97	0.97	1.0		
	Gentamicin	256	2.00	0.00	1.0	additive
	THIPMC	0.97	0.97	1.0		
CL6	Ampicillin	256	2.00	0.00	0.25	Synergistic
	THIPMC	3.90	0.97	0.25		
	Gentamicin	256	2.00	0.00	0.25	Synergistic
	THIPMC	3.90	0.97	0.25		
CL9	Ampicillin	128	32.0	0.12	1.12	indifferent
	THIPMC	15.6	15.6	1.00		
	Gentamicin	256	32.0	0.12	1.12	indifferent
	THIPMC	15.6	15.6	1.00		
CL19	Ampicillin	256	1.00	0.00	0.25	Synergistic
	THIPMC	1.95	0.49	0.25		
	Gentamicin	256	0.5	0.00	0.125	Synergistic
	THIPMC	1.95	0.25	0.125		

[a]7,9,2',4'-tetrahydroxy-8-isopentenyl-5-methoxychalcone (THIPMC)

[b]MIC required to inhibit 90% of strains.

[c]The checkboard test was performed as previously described [Jókay *et al*., 2001].

[d]FIC indexes were interpreted as synergy ≤0.5; partial synergy FIC >0.5 but <1; additive FIC=1.0; indifferent >1 and <4, and antagonistic when values were ≥4.0 [Mates *et al*., 1982].

were evaluated using MRSA [Hatano *et al*., 2000]. For these assays, a standard inoculum of approximately 1×10^6 CFU/mL of an overnight culture was used. All agents were administered at their MIC concentrations. Combinations of antimicrobial agents and THIPMC were also evaluated. A test plate containing only MHB was inoculated and served as a control. Counts of viable strains were carried out at different times up for up to 4 h at 37°C. Rates and extents of killing were determined by plotting viable colony counts (CFU/mL) against time in MHA.

Combined effects of THIPMC with AM or GM. The antibacterial effects of combined treatments were assessed using the checkerboard test [Shahverdi *et al*., 2007]. FIC indexes were calculated using the following formula: FIC =($MIC_{Drug\ A}$ in combination/$MIC_{Drug\ A}$ alone)+($MIC_{Drug\ B}$ in combination/$MIC_{Drug\ B}$ alone). Briefly, bacterial cells (1×10^8 CFU/mL) were inoculated into MHB and dispensed at 10 μL/well into 96-well microtiter plates. MICs were determined by serial two-fold dilutions of THIPMC and/or antibiotics. After 16 h of incubation at 37°C, minimum concentrations that prevented the growths of test organisms were determined (defined as MICs). MIC values were determined using three independent assays and FIC indexes were calculated from the FIC indexes of THIPMC and antibiotics. FIC indexes indicate synergy when ≤0.5; partial synergy for FIC values of >0.5 but <1, an additive effect for FIC values of =1.0, and indifferent effect for FIC values of >1 but <4, and an antagonistic effect for FIC values of ≥4.0 [Mates *et al*., 1982].

Results and Discussion

Identification of THIPMC. The ^{1}H-NMR spectrum had resonances due to the presence of two tertiary methyl groups at δ 1.76 and 1.63 and a methoxy group at δ 3.89. The signals of 1 for a set of trans-coupled olefinic protons δ 8.16 and 7.98 (1H, d, *J*=15.6 Hz, each) confirmed the existence of the chalcone and the typical pattern of a coupling group of 1,3,4-trisubstituted benzene was

Table 3. Synergic effect of THIPMC, AM, PE, CL and CE against VRE

Strains	Agent	MIC[b] (μg/mL)		FIC (μg/mL)	FICI[d]	Outcome
		Alone	Combination[c]			
VRE24	Ampicillin	256	32	0.12	1.125	indifferent
	[a]THIPMC	15.6	15.6	1.00		
	Penicillin	512	32	0.06	1.062	indifferent
	THIPMC	15.6	15.6	1.00		
	Clindamycin	512	32	0.06	1.062	indifferent
	THIPMC	15.6	15.6	1.00		
	Cephalothin	1024	32	0.03	1.031	indifferent
	THIPMC	15.6	15.6	1.00		
CL186	Ampicillin	128	8	0.06	0.312	Synergistic
	THIPMC	15.6	3.9	0.25		
	Penicillin	512	32	0.06	1.250	indifferent
	THIPMC	15.6	15.6	1.00		
	Clindamycin	128	8.0	0.06	0.312	Synergistic
	THIPMC	15.6	3.9	0.25		
	Cephalothin	512	32	0.06	1.250	indifferent
	THIPMC	15.6	15.6	1.00		
CL189	Ampicillin	512	32	0.06	2.062	indifferent
	THIPMC	7.8	15.6	2.00		
	Penicillin	1024	16	0.01	1.015	indifferent
	THIPMC	7.8	7.8	1.00		
	Clindamycin	512	16	0.03	1.031	indifferent
	THIPMC	7.8	7.8	1.00		
	Cephalothin	1024	16	0.01	1.015	indifferent
	THIPMC	7.8	7.8	1.00		

Table 3. Continued

Strains	Agent	MIC [b] (μg/mL)		FIC (μg/mL)	FICI[d]	Outcome
		Alone	Combination[c]			
CL232	Ampicillin	256	32.0	0.12	1.125	indifferent
	[a]THIPMC	15.6	15.6	1.00		
	Penicillin	1024	32.0	0.03	1.031	indifferent
	THIPMC	15.6	15.6	1.00		
	Clindamycin	512	16.0	0.03	0.531	partial synergy
	THIPMC	15.6	7.8	0.5		
	Cephalothin	1024	32.0	0.03	1.031	indifferent
	THIPMC	15.6	15.6	1.00		
CL233	Ampicillin	512	8.0	0.01	0.515	partial synergy
	THIPMC	7.8	3.9	0.5		
	Penicillin	1024	16.0	0.01	1.015	indifferent
	THIPMC	7.8	7.8	1.00		
	Clindamycin	512	15.0	0.03	1.032	indifferent
	THIPMC	7.8	7.8	1.00		
	Cephalothin	1024	16.0	0.01	1.015	indifferent
	THIPMC	7.8	7.8	1.00		
CL238	Ampicillin	512	32.0	0.06	1.306	indifferent
	THIPMC	15.6	15.6	1.00		
	Penicillin	1024	32.0	0.03	1.031	indifferent
	THIPMC	15.6	15.6	1.00		
	Clindamycin	512	32.0	0.03	1.062	indifferent
	THIPMC	15.6	15.6	1.00		
	Cephalothin	1024	32.0	0.03	1.031	indifferent
	THIPMC	15.6	15.6	1.00		

[a]7,9,2',4'-tetrahydroxy-8-isopentenyl-5-methoxychalcone (THIPMC)
[b]MIC required to inhibit 90% of strains.
[c]The checkboard test was performed as previously described [Jókay *et al*., 2001].
[d]FIC indexes were interpreted as synergy ≤0.5; partial synergy FIC >0.5 but <1; additive FIC=1.0; indifferent >1 and <4, and antagonistic when values were ≥4.0 [Mates *et al*., 1982].

observed at δ 7.53 (1H, d, *J*=8.0 Hz, H-6'), 6.50 (1H, d, *J* =2.0 Hz, H-3') and 6.45 (1H, d, *J*=8.0, 2.0 Hz, H-5'). In the ^{13}C-NMR spectrum, the signals of five hydroxyl bonded aromatic carbons (δ 166.5, 162.6, 162.0, 161.9 and 159.7), one carbonyl carbon (δ 193.8) and one isoprenyl bonded carbon (δ 109.3) were observed. In the HMBC spectrum, the isoprenyl proton signals H-1" (δ 3.28) and H-2" (δ 5.24) showed ^{1}H-^{13}C long-range correlation with C-8 (δ 109.3). On the basis of these observations, the structure of 1 was deduced as a prenylated chalcone derivative. Considering the down field shift of the carbon signal for C-8 (δ 109.3) and the correlation with the protons C-1" (δ 3.28) and C-2" (δ 5.24) of the isoprenyl group in the HMBC spectrum, the position of the isoprenyl group was determined to be at C-8. By comparison of ^{1}H- and ^{13}C-NMR spectrum data with the literature values [Lee *et al*., 2007], compound was characterized as **7**,9,2',4'-tetrahydroxy-8-isopentenyl-5-methoxychalcone (THIPMC, Fig. 1).

The effects of THIPMC on MRSA or VRE. The antimicrobial effects of THIPMC on human pathogenic bacteria, including clinical isolates of antibiotic-resistant bacteria, were investigated and described as the MIC. we measured the MICs of it with clinically isolated MRSA and VRE strains. In this result, we observed MIC values of 0.97 to 15.6 μg/mL with the MRSA strains and 7.8 to 15.6 μg/mL with VRE strains tested (Table 1). THIPMC were able to inhibit the growth of these MRSA strains than VRE strain, at relatively low concentrations, however, effect of antibacterial activity of THIPMC in VRE exhibited

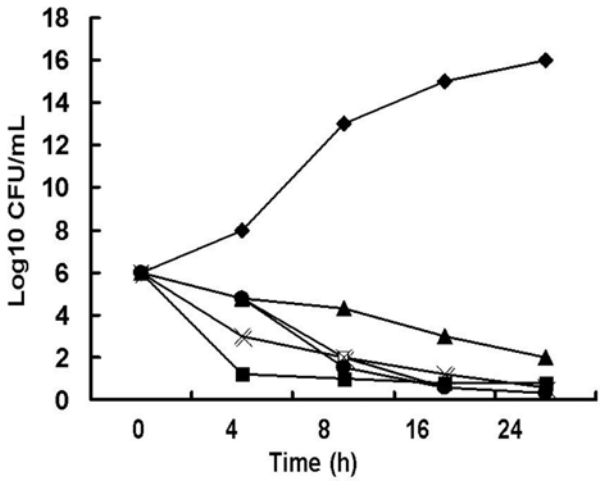

Fig. 2. Time-kill curve showing the in vitro bactericidal effect of THIPMC on VRE24 strain. Symbol: ◆, control; □, Penicillin+THIPMC; ▲, Clindamycin+THIPMC; ⧧, Cephalothin+THIPMC; ■, Ampcillin+THIPMC; ●, THIPMC. MIC of the VRE24 strain: Penicillin 512 μg/mL; Clindamycin 512 μg/mL; Cephalothin 1024 μg/mL <; Ampcillin 256 μg/mL; THIPMC 15.6 μg/mL.

less activity than MRSA (Table 1). Thus, THIPMC are effective on MRSA at similar concentrations. Table 2 shows the MICs of ampicillin, gentamicin and THIPMC against MRSA strains. Also, against VRE strains, the MIC of vancomycin was very high, higher than 512 μg/mL (data not showed). Of the 6 strains are clinically isolated VRE strains that showed very high MICs of cephalothin (CE), clindamycin (CL), ampicillin (AM), and penicillin (PE) (Table 3). The MICs of AM or GM ranged from 128 to 256 μg/mL, 256 to 512 μg/mL for MRSA strain (Table 2). And against VRE with *vanA* gene strains, the MICs for CE, CL, AM, PE ranged from 512 to 1024 μg/mL, 128 to 512 μg/mL, 128 to 512 μg/mL, 512 to 1024 μg/mL, respectively (Table 3). These antimicrobial agents are basically ineffective on these MRSA or VRE strains. In the synergic effect test results, the FIC indexes of THIPMC, AM, GE combination ranged from 0.125 to 1.120 μg/mL in MRSA strain (Table 2), 0.313 to 2.063 μg/mL, detecting against VRE strain (Table 3). For the effects of drug combinations, we investigated the antimicrobial killing activity of THIPMC against MRSA strains. Fig. 2 showed the test results of the time-kill curve against bactericidal activities. In the result, THIPMC alone, the rate of killing CFU/mL decreased after 4 h, also the combination of the antibiotic and THIPMC increased rate of killing after 4 h. Thus the drug combinations exhibited a bactericidal activity. Meanwhile, more than 6,500 different flavonoids have been identified from plant sources, of which at least 400 appear to be isoprenoid-substituted. Flavonids are known to exhibit a number of beneficial properties for human health due to their interactions with a number of cellular targets; these targets include anti-oxidant and free-radical scavenger activities, as well as anti-inflammatory, antiviral and especially antimicrobial activity [Di Pietro *et al*., 2002]. The substitution of the flavonoid ring system an isoprenoid moiety increases their lipophilicity and affinity to biological membranes [Chi *et al*., 2001]. Sohn *et al*. [2004] reported that antimicrobial and cytotoxic activity of 18 prenylated flavonoids isolated from medicinal plants. Our results revealed a similar MICs against MRSA. Here, we report the clear importance of isoprenoid moiety substitutions in flavonoids as a source of potent antibacterial activity. Our findings indicate that THIPMC is uniformly active against clinical isolates of MRSA and VRE. Further studies are needed for testing mechanisms of action of THIPMC on MRSA.

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