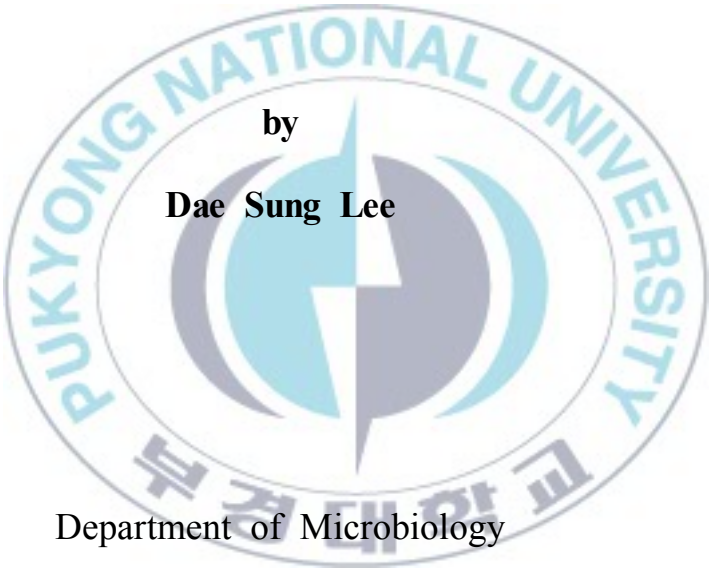
Thesis for the Degree of Doctor of Philosophy

Isolation and Characterization of Anti-MRSA (Methicillin-Resistant *Staphylococcus aureus*) Substances from Marine Organisms



by

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February 2009

Isolation and Characterization of Anti-MRSA (Methicillin-Resistant *Staphylococcus aureus*) Substances from Marine Organisms

해양 생물체로부터 MRSA에 대한 항균물질의 분리 및 특성

Advisor: Prof. Won Jae Lee

by

Dae Sung Lee

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A dissertation

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Abstract

It has been attempting to discover a compound evidencing antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) from marine organisms. Anti-MRSA activities of dieckol isolated from *Ecklonia stolonifera* and 1-acetyl-beta-carboline isolated from *Pseudomonas* sp. UJ-6 were investigated. In order to identify an anti-MRSA substance from the solvent soluble fractions of *E. stolonifera* and *Pseudomonas* sp. UJ-6, a high performance liquid chromatography (HPLC), ^{1}H-, ^{13}C-, and 2D-NMR (nuclear magnetic

resonance) and LC-mass analyses were performed. ^{1}H- and ^{13}C-NMR spectroscopic assignment analyses were performed using correlation spectroscopy (COSY), gradient heteronuclear single quantum coherence (gHSQC), and gradient heteronuclear multiple bond coherence (gHMBC).

The dieckol purely isolated from *E. stolonifera* exhibited the antibacterial activity against methicillin-susceptible *S. aureus* (MSSA) and MRSA. The minimum inhibitory concentrations (MICs) of dieckol were determined to range 32 to 64 μg/ml against the standard MSSA and MRSA strains. Furthermore, less than 16 μg/ml of dieckol obviously reversed the high level resistance of MRSA to ampicillin and penicillin. The MICs of ampicillin against two standard MRSA strains were dramatically dropped from 512 to 0.5 μg/ml in combination with 1/4 MIC of dieckol (16 μg/ml). The fractional inhibitory concentration (FIC) indices of ampicillin and penicillin were from 0.066 to 0.266 in combination with 8 or 16 μg/ml of dieckol against all MRSA strains tested, indicating synergistic effect in dieckol- ampicillin or penicillin combination against MRSA.

It was isolated a marine bacterium, *Pseudomonas* sp. UJ-6, which produced a bactericidal antibiotics against MRSA. This strain was tested by examining its morphological and biochemical properties and

16S rDNA sequencing analysis for identification. The strain UJ-6 was identified to be *Pseudomonas* genus. *Pseudomonas* sp. UJ-6 relatively grows well in the range of 20-30℃, pH 5.0-8.0 and NaCl 0-3%. The culture broth and its ethyl acetate extract exhibited a bactericidal activity against MRSA. An abnormal cell lysis of MRSA was observed when MRSA cells were grown in the presence of extract. The extract kept over 95% of its activity after heat treatment for 15 min at 121℃, suggesting that a thermal stable antibiotic is produced by *Pseudomonas* sp. UJ-6, even tough most known antibiotics are unstable to thermal stress. 1-acetyl-beta-carboline isolated from *Pseudomonas* sp. UJ-6 showed an antibacterial activity against MRSA strains tested with minimum inhibitory concentrations ranging from 32 to 64 μg/ml.

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List of Abbreviations

ANT 4'	aminoglycoside adenylyltransferase 4'
CDC	Centers for Disease Control and Prevention
COSY	correlation spectroscopy
EGCg	epigallocatechin gallat
FIC	fractional inhibitory concentration
gHSQC	gradient heteronuclear single quantum coherence
gHMBC	gradient heteronuclear multiple bond coherence
GISA	glycopeptide-intermediate *Staphylococcus aureus*
HPLC	high performance liquid chromatography
KCCM	Korea Culture Center of Microorganisms
KCTC	Korean Collection for Type Cultures
KORDI	Korea Ocean Research and Development Institute
MH	Mueller-Hinton
MIC	minimum inhibitory concentration
MRSA	methicillin-resistant *Staphylococcus aureus*
MSSA	methicillin-susceptible *Staphylococcus aureus*
NCEs	new chemical entities
NMR	nuclear magnetic resonance
ODS	octadecylsilyl silica
PABA	para-aminobenzoic acid
PBP	penicillin-binding protein
SCC*mec*	staphylococcal chromosomal cassette *mec*
TEM	transmission electron micrograph
VISA	vancomycin-intermediate *Staphylococcus aureus*
VRSA	vancomycin-resistant *Staphylococcus aureus*

Chapter 1

General Introduction of MRSA (Methicillin-Resistant *Staphylococcus aureus*)

1. Emerging of antibiotic resistance

When the first antibiotics such as penicillin began making their way into clinical use, they were hailed as miracle drugs. By killing the bacteria that cause many of humankind's worst infectious diseases, such as tuberculosis and pneumonia, they saved countless lives. But not all miracles last forever (Travis, 1994). Various microorganisms have survived for many years by their ability to adapt to antimicrobial agents (Levy, 1998). In the presence of drugs, microorganisms have been evolved sophisticated mechanisms to inactivate these compounds (e.g. by pumping out compounds, mutating residues required for the compound to bind, etc.), and they do so at a rate that far exceeds the pace of new development of drugs. In addition, some resistant bacteria are able to transfer copies of DNA that codes for a mechanism of resistance to other bacteria, thereby conferring resistance to their neighbors, which then are also able to pass on the resistant gene.

2. Introduction and antibiotic resistance of *Staphylococcus aureus*

S. aureus (Fig. 1) is a facultatively anaerobic, Gram-positive coccus, which appears as grape-like clusters when viewed through a microscope and has large, round, golden-yellow colonies, often with hemolysis, when grown on blood agar plates. *S. aureus* is the most common cause of staph infections and frequently found in the nose and skin of person (Kluytmans *et al*., 1997). *S. aureus* can cause a range of illnesses from minor skin infections, such as folliculitis and furunculosis, to deep-seated and life-threatening conditions including erysipelsa, deep abscesses, osteomyelitis, pneumonia, sepsis and endocrditis (Lowy, 1998; Guignard *et al.*, 2005; Park *et al.*, 2007).

MRSA have acquired successively a number of resistance determinants by acquisition of plasmids and transposons. Resistance of staphylococci to oxacillin and other β-lactam antibiotics is mediated by a specific penicillin-binding protein (PBP2a), which has a decreased affinity for β-lactam antibiotics. PBP2a is encoded by the *mec*A gene, located on a mobile genetic element designated staphylococcal chromosomal cassette *mec* (SCC*mec*), which is present on the chromosome of all MRSA isolates. Clinical MRSA has been defined as *S. aureus* having the *mec*A gene with a MIC of oxacillin

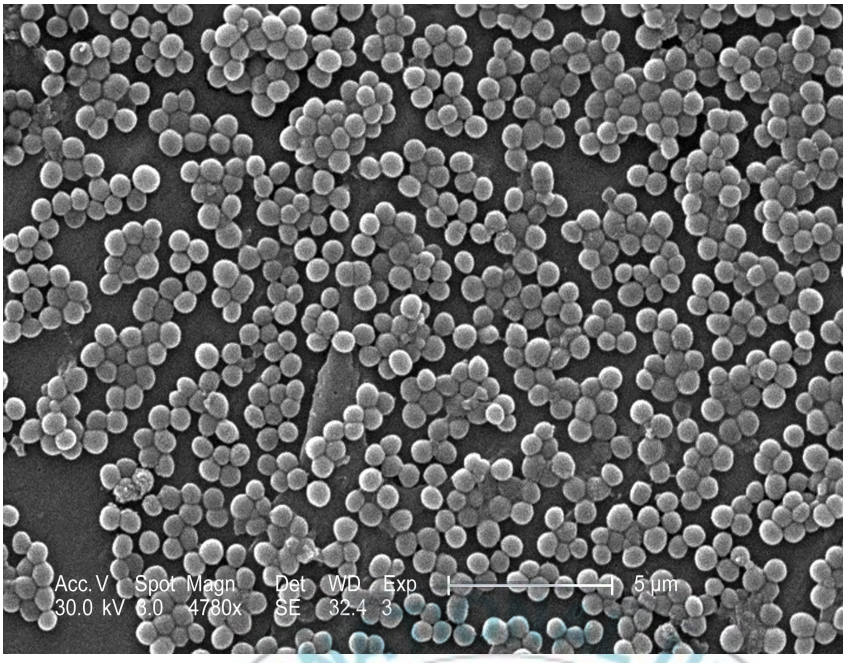


Fig. 1. Microscopic image of *Staphylococcus aureus*.

higher than 4 μg/mL (Ito *et al*., 2001). However, some clinical isolates has been described as *mec*A positive and oxacillin susceptible with MICs to oxacillin 2 μg/mL or less (Hososaka *et al*., 2007; Swenson *et al*., 2007; Witte *et al*., 2007).

3. Mechanisms of bacterial resistance to antibiotics

When bacteria are exposed by antibiotics, most of cells will affect and to be death. However, some of cells that acquired mechanism of antibiotic resistance will survive and reproduce, and the new population will be drug-resistant. Many bacteria now exhibit multidrug resistance, including staphylococci, enterococci, gonococci, streptococci, and others. The four main mechanisms by which microorganisms exhibit resistance to antimicrobials are:

(1) Drug inactivation or modification: e.g. enzymatic deactivation of Penicillin G in some penicillin-resistant bacteria through the production of β-lactamases.
(2) Alteration of target site: e.g. alteration of PBP - the binding target site of penicillins - in MRSA and other penicillin-resistant bacteria.
(3) Alteration of metabolic pathway: e.g. some sulfonamide-resistant

bacteria do not require para-aminobenzoic acid (PABA), an important precursor for the synthesis of folic acid and nucleic acids in bacteria inhibited by sulfonamides. Instead, like mammalian cells, they turn to utilizing preformed folic acid.

(4) Reduced drug accumulation: by decreasing drug permeability and/or increasing active efflux (pumping out) of the drugs across cell membrane.

Antibiotic resistance of *S. aureus* was almost unknown when penicillin was first introduced in 1943; indeed, the original petri-dish on which Alexander Fleming observed the antibacterial activity of the penicillium mold was growing a culture of *S. aureus*. However, today, *S. aureus* has become resistant to many commonly used antibiotics. The increasing antibiotic resistance of *S. aureus* has become a serious problem, especially in the nosocomial- and community-associated infection (Schaberg *et al*., 1991; Witte, 1999; Levy, 2005). By 1950, 40% of hospital *S. aureus* isolates were penicillin resistant; and by 1960, this had risen to 80% (Finland, 1979; Chambers, 2001). By 1960, many hospitals had outbreaks of virulent multi-resistant *S. aureus*. These were overcome with penicillinase-stable penicillins (methicillin, oxacillin, cloxacillin and flucloxacillin), but only two years later, the first case of methicillin-resistant *S. aureus* (MRSA)

was reported in England (Jevons, 1961). Recently, MRSA has been the most problematic gram-positive bacterium in public health because it has become resistant to almost all available antibiotics except vancomycin and teicoplanin (Witte, 1999 Isnansetyo and Kamei, 2003).

The mechanism of resistance to methicillin is mediated via the *mec* operon, part of the staphylococcal cassette chromosome *mec* (SCC*mec*). Resistance is conferred by the *mec*A gene, which codes for an altered penicillin-binding protein (PBP2a or PBP2') that has a lower affinity for binding β-lactams (penicillins, cephalosporins and carbapenems). This allows for resistance to all β-lactam antibiotics and obviates their clinical use during MRSA infections. MRSA has become resistant to almost all available antibiotics except vancomycin and teicoplanin. As the result, the glycopeptide, vancomycin, is often deployed against MRSA. The most important resistance mechanisms and resistance genes associated with MRSA are summarized in Table 1.

Aminoglycosides such as kanamycin, gentamicin, and streptomycin were once effective against staphylococcal infections until the organism evolved mechanisms to destroy the aminoglycosides action, which occurs via protonated amine and/or hydroxyl interactions with the ribosomal RNA of the bacterial 30S ribosome (Carter *et al.*, 2000).

Table 1. Resistance mechanisms and resistance genes in multiply-resistant *Staphylococcus aureus* (Witte, 1999)

Antibacterial substances	Type of mechanism	Mechanism	Resistance gene(s)	Genetic localization
Penicillins	inactivation	β-lactamase(serotypes A-D)	*blaZ*	plasmids(19-33kb)Tn552(Tn4002, Tn4001)
All β-lactams	new target with reduced affinity	PBP2a	*mecA*	Tn4291
Aminoglycosides	drug modification	aminoglycosidases AAC6'/APH2"	*aacA-aphD*	Tn4001, also integrated into plasmid (conjugative)
		ANT4,4"	*aaD*	plasmids
		APH3"	*aphA*	plasmids, chromosome
		ANT3"-1	*aadE*	Tn5404,Tn5405, integrated into plasmids or the chromosome
Macrolides/	modification of nautral target	23S rRNA methylase	*ermA*	Tn554
lincosamines/			*ermB*	plasmids of pl 258 type
B-streptogramins(MLS_B)			*ermC*	plasmids of pE 184 type
Macrolides	active efflux	ABC-carrier	*msrA*	plasmids (pUL 5050)
Streptogramins(A)	drug modification	acetyltransferases	*vat, vatB*	plasmids
	efflux	ABC-carrier	*vha*	plasmids
	inactivation	hydrolysis	not analysed in detail	plasmids
Streptogramins(B)	see MLS			
Tetracyclines	active efflux	class K and L systems	*tetK*	plasmids
			tetL	plasmids
	sequestration of antibiotic	class M system	*tetM*	Tn 916
Rifampicin	target alteration	mutation in B-subunit of RNA polymerase	*rif*	chromosomal mutation
Fusidic acid	reduced influx	mechanism unknown		plasmids
Quinolones	target alteration	mutations in the A-subunits of gyrase and topoisomeraseⅣ	*gyrA, grlA*	chromosomal mutations
	active efflux	proton pump		plasmids
Mupirocin	new target with reduced affinity	resistant isoleucyl-t-RNA synthetase	*mupA*	plasmids (30-35kb)
Trimethoprim	new target with reduced affinity	resistant dihydrolate reductase	*dfrA*	Tn4003, integrated into plasmids (conjugative)
Glycopeptides	overproduction of physiological target	trapping effect	*VanA*	probably chromosomal

There are three main mechanisms of aminoglycoside resistance mechanisms which are currently and widely accepted: Aminoglycoside modifying enzymes, ribosomal mutations, and active efflux of the drug out of the bacteria.

Aminoglycoside modifying enzymes are enzymes that inactivate the aminoglycoside by covalently attaching either a phosphate, nucleotide, or acetyl moiety to either the amine and/or alcohol functionality of the antibiotic; thus rendering the antibiotic through sterics or lack of charge, ineffective in ribosomal binding affinity. In *S. aureus,* the best characterized aminoglycoside modifying enzyme is ANT 4' IA (Aminoglycoside adenylyltransferase 4' IA). This enzyme has been solved by X-ray crystallography. The enzyme is able to attach an adenyl moiety to the 4' hydroxyl group of many aminoglycosides including kamamycin and gentamicin (Sakon, 1993).

Glycopeptide resistance is mediated by acquisition of the *van*A gene. The *van*A gene originates from the enterococci and codes for an enzyme that produces an alternative peptidoglycan to which vancomycin will not bind.

4. Prevalence of MRSA

MRSA is often referred to in the press as a "superbug". In the past decade or so the number of MRSA infections in the United States has increased significantly (Fig. 2). A 2007 report in Emerging Infectious Diseases, a publication of the Centers for Disease Control and Prevention (CDC), estimated that the number of MRSA infections treated in hospitals doubled nationwide, from approximately 127,000 in 1999 to 278,000 in 2005, while at the same time deaths increased from 11,000 to more than 17,000 (Klein *et al*., 2007). Another study led by the CDC and published in the October 17, 2007 issue of the Journal of the American Medical Association estimated that MRSA would have been responsible for 94,360 serious infections and associated with 18,650 hospital stay-related deaths in the United States in 2005 (Klevens *et al.*, 2007). The UK Office for National Statistics reported 1,629 MRSA-related deaths in England and Wales during 2005, indicating a MRSA-related mortality rate half the rate of that in the United States for 2005, even though the figures from the British source were explained to be high because of "improved levels of reporting, possibly brought about by the continued high public profile

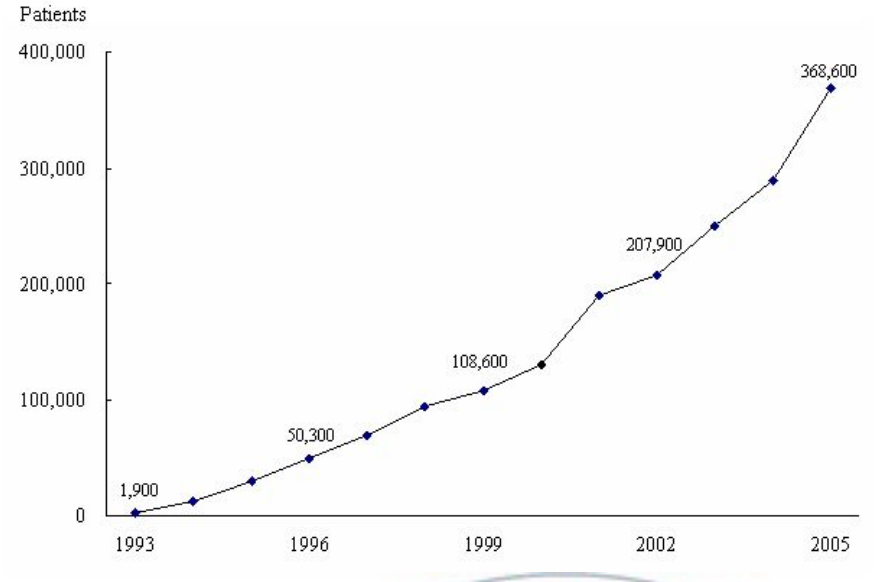


Fig. 2. Hospital stays with MRSA infections 1993-2005 (CDC, 2007).

of the disease" during the time of the 2005 United Kingdom General Election. MRSA is thought to have caused 1,652 deaths in 2006 in UK up from 51 in 1993 (Fig. 3, UK Office for National Statistics, 2007).

It has been argued that the observed mortality increased among MRSA-infected patients may be the result of the increased underlying morbidity of these patients. Several studies, however, including one by Blot and colleagues, that have adjusted for underlying disease still found MRSA bacteremia to have a higher attributable mortality than methicillin-susceptible *Staphylococcus aureus* (MSSA) bacteremia (Blot *et al.*, 2002). While the statistics suggest a national epidemic growing out of control, it has been difficult to quantify the degree of morbidity and mortality attributable to MRSA. A population-based study of the incidence of MRSA infections in San Francisco during 2004-5 demonstrated that nearly 1 in 300 residents suffered from such an infection in the course of a year and that greater than 85% of these infections occurred outside of the health care setting (Liu *et al.*, 2008). A 2004 study showed that patients in the United States with *S. aureus* infection had, on average, three times the length of hospital stay (14.3 vs. 4.5 days), incurred three times the total cost ($48,824 vs $14,141), and experienced five times the risk of in-hospital death

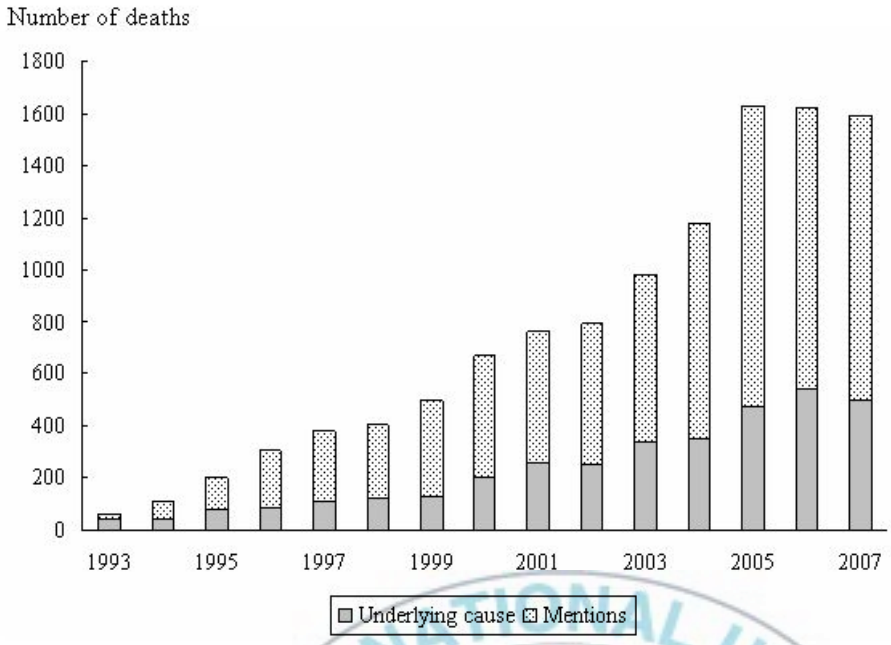


Fig. 3. Number of death certificates mentioning *Staphylococcus aureus* by methicillin resistance, England and Wales (UK Office for National Statistics, 2007).

(11.2% vs 2.3%) than patients without this infection (Noskin *et al.*, 2005). In a meta-analysis of 31 studies, Cosgrove *et al.* (2005) concluded that MRSA bacteremia is associated with increased mortality as compared with MSSA bacteremia (odds ratio = 1.93; 95% CI = 1.93±0.39) (Hardy *et al.*, 2004) In addition, Wyllie *et al.* reported a death rate of 34% within 30 days among patients infected with MRSA, compared to the death rate of 27% seen among MSSA-infected patients (Wyllie *et al.*, 2006).

5. Strategies for overcoming antibiotic resistance of bacteria

As the problem of bacterial resistance has increased, the outlook for the use of antibacterial drugs in the future is uncertain. Thus, the development of new drugs or alternative therapies is required, which is active against resistant bacteria (Nascimento *et al.*, 2000). In response to bacterial resistance, many researchers have studied for alternative antibiotics and therapies.

5.1. Synthesis of new chemical entities

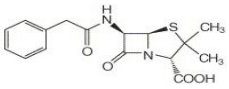
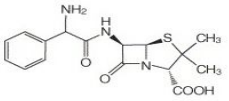
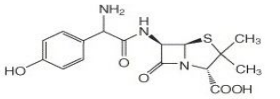
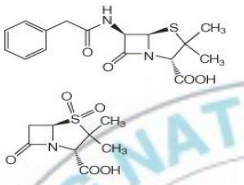
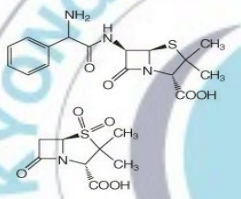
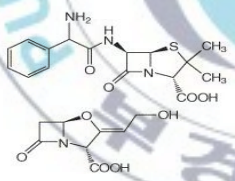
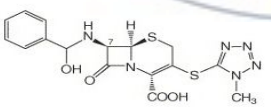
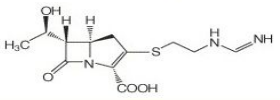
Many researchers have studied to develop new antibiotics, either synthetic or natural. They are synthesizing new chemical entities (NCEs) in the search for new β-lactams, such as cephalosporin-based antibiotics (Lloyd, 1998a), dithiocarbamate carbapenems and 2-metabiphenyl carbapenems (Lloyd, 1998b).

The concept of anti-PBP2a β-lactams was pursued. Compassionate use of the older molecules is being employed in cases of difficult-to-treat MRSA or GISA (glycopeptide-intermediate *S. aureus*) infections (Nicolas *et al.*, 1993; Hiramatsu *et al.*, 1997). In parallel, drug chemistry succeeded in generating semi-synthetic drugs with greatly improved PBP2a affinity.

β-lactams with anti-MRSA activity were described in all of the three classes of penams, cephems and carbapenems (Table 2). However, novel anti-MRSA molecules mainly cluster among cephems and carbapenems (Table 3 and Table 4). This could be related to the difficulty of modifying functional groups at the ring fused to the β -lactam ring in penam molecules.

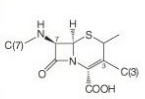
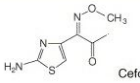
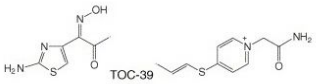
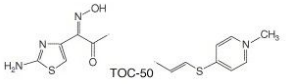
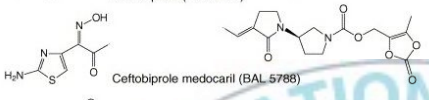
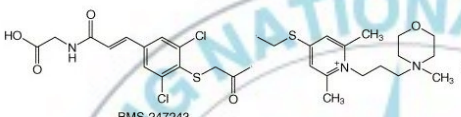
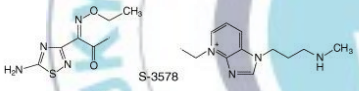
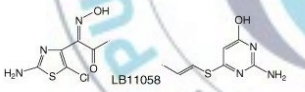
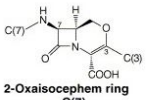
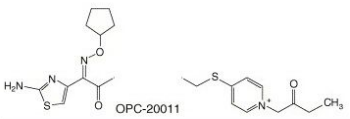
Despite these developmental hindrances, clinically useful anti-MRSA β-lactams are likely to be imminent. Because they mimic an adduct (D-ala-D-ala) unique to the bacterial world, β-lactams can be given in

Table 2. Characteristics of classic β-lactams with anti-MRSA activity (Guignard *et al.*, 2005)

		MIC_{90}[a] [μg/ml]	IC_{50}[b] [μg/ml]	β-lactamase stability
Penicillin G[c]		>32	14	Unstable
Ampicillin[c]		>32	4	Unstable
Amoxicillin[c]		>32	4	Unstable
Penicillin G Sulbactam		8	14	Stable
Ampicillin Sulbactam		16	4	Stable
Amoxicillin Clavulanate		16	4	Stable
Cefamandole		≤16	8	Relatively stable
Imipenem		32	68	Stable

a, MIC of the compound at which 90% of MRSA were inhibited.
b, Concentration inhibiting 50% of [3H] penicillin-labelling affinity for PBP2a.
c, Inactive against penicillinase-producing isolates, unless combined with sulbactam or clavulanate.

Table 3. Characteristics of novel anti-MRSA cephems (Guignard *et al.*, 2005)

Cephem ring  C(7) C(3)			MIC$_{90}$[a] [μg/ml]	IC$_{50}$[b] [μg/ml]	β-lactamase stability
	Cefoselis (FK037)		25	3.0	Stable
	TOC-39		3.13	ND	ND
	TOC-50		3.13	ND	ND
	Ceftobiprole (BAL 9141)		≤4	0.5	Stable
	Ceftobiprole medocaril (BAL 5788)				
	BMS-247243		5	0.7	Relatively stable
	RWJ-54428 (MC-02 479)		2	0.7	Stable
	S-3578		4	4.42	Stable
	LB11058		1	0.8	Stable
	CB-181963 (CAB-175)		2–4	1	Stable
 2-Oxaisocephem ring C(7) C(3)					
	OPC-20011		6.25	2.0	ND

a, MIC of the compound at which 90% of MRSA were inhibited.
b, Concentration inhibiting 50% of [3H] penicillin-labelling affinity for PBP2a. ND, not determined.

Table 4. Characteristics of novel anti-MRSA carbapenems (Guignard *et al*., 2005)

Carbapenem ring C(1) C(2)	MIC_{90} [a] [μg/ml]	IC_{50} [b] [μg/ml]	DHP-I stability
∅ L-695 256	2	1.2	ND
SM-17466	3.15	5.9	Stable
SM-232724	2	1.3	Relatively stable
BO-3482	6.25	3.8	Unstable
J-111 225	4	2.5	Stable
CS-023	4	5.3	ND

a, MIC of the compound at which 90% of MRSA were inhibited.

b, Concentration inhibiting 50% of [3H] penicillin-labelling affinity for PBP2a. ND, not determined.

large doses to humans. Thus, new anti-PBP2a β-lactams should provide a most appropriate response to the increase in multi-resistant MRSA, including the recent glycopeptide-resistant isolates.

5.2. Screening NCEs for new antibiotics

An alternative strategy to synthesis NCEs is their identification by screening natural sources such as bacteria, fungi and plants. Especially, screening bacteria from marine ecosystems, such as the deep sea floor, could lead to the isolation of new antibiotics (Tan *et al*., 2000). The chemical compounds produced by marine bacteria are less well known than those of their terrestrial counterparts. Many such organisms produce marine natural products that possess unique structural features as compared to terrestrial metabolites (Larsen *et al*., 2005). Among them, a few and novel antibiotics from marine microorganisms have been reported, including loloatins from *Bacillus* (Gerard *et al.*, 1999), agrochelin and sesbanimides from agrobacterium (Acebal *et al*., 1998; Acebal *et al*., 1999), pelagiomicins from *Pelagiobacter variabilis* (Imamura *et al*., 1997), δ-indomycinone from a *Streptomyces* sp. (Biabani *et al*., 1999), and dihydrophencomycin methyl ester from *Streptomyces* sp. (Pusecker *et al*., 1997). Screening of antibiotics has been traditionally performed with Actinomyces such

as *Streptomyces* species. Therefore, to screen novel secondary metabolites such as antibiotics from marine bacteria are attracting attention since marine bacteria have been believed that their physiological and genetical characteristic are different compared to soil bacteria such as *Streptomyces* sp..

5.3. Synergistic effect by combination with commercial antibiotics

During the last 20 years, the number of new drugs has been declining. Concomitantly, resistance is rising to high levels among bacterial pathogens. This means that the rate of loss of efficacy of old antibiotics is outstripping their replacement with new ones for many species of pathogenic bacteria (Hancock, 2007). It is not uncommon for the effect of two chemicals on an organism to be greater than the effect of each chemical individually, or the sum of the individual effects. The presence of one chemical enhances the effects of the second. It is designed to either improve efficacy through synergistic action of the agents, or to overcome bacterial resistance.

5.4. The genomics revolution

The complete sequencing of the genomes of many pathogenic bacteria has led to an explosion in knowledge about these organisms (Monaghan and Barrett, 2006). Genomics is used to select potential antibacterial targets (Sakharkar *et al*., 2004; Arcus *et al*., 2006) and can also be used to provide insights into, for example, pathogenesis (Field *et al*., 2004; Polissi and Soria, 2005) and antibiotic resistance (Black and Hodgson, 2005; Fournier *et al*., 2006). An antibacterial target may be the receptor of a ligand in a bacterial molecule, a specific function of a bacterial molecule such as an enzyme or a metabolic pathway. Libraries of compounds may be screened at this time to find a lead molecule that, for example, inhibits the enzymatic function of the target. Further development includes searching for antibacterial activity of the molecule. Several lead compounds are chosen and lead optimization then proceeds to complete the preclinical programme, which is required before entry into clinical trials. In the case of antibiotic discovery, this approach and many other similar ones have been and are being attempted (Freiberg and Brotz-Oesterhelt, 2005). However, so far, no marketed antibiotics have reached the marketplace via the genomics route (Mills, 2006). The reason for this failure is not entirely clear. Peptide deformylase is one

target that has been identified with the assistance of genomic information (Yuan and White, 2006), and inhibitors of this enzyme have been developed, which have entered into human clinical trials (Clements *et al*., 2002). Unfortunately, these inhibitors seem to generate mutants at a high rate (Margolis *et al*., 2000; Apfel *et al*., 2001) and hence development has not proceeded as expected.

5.5. Bacteriophages

Bacteriophages and their fragments (Borysowski *et al*., 2006) kill bacteria. It is estimated that every 2 days, half of the world's bacterial population is destroyed by bacteriophages (Hendrix, 2002). Bacteriophages have been used as antibacterials in humans in some countries of the world (Sulakvelidze *et al*., 2001). Potential advantages of this approach are that the mechanism of action is likely to be completely different to current antimicrobials. Disadvantages are that quality control and standardization are difficult. Also, when used systemically in patients, phages are likely to be immunogenic and may induce neutralizing antibodies (Dabrowska *et al*., 2005). Furthermore, massive bacterial lysis might lead to toxic shock (Matsuda *et al*., 2005). One approach that has been developed to address this problem is lysis-deficient phages, which can still kill

bacteria (Matsuda *et al*., 2005). The development of phage gene products is another potential route for new antibacterials. Phage lysins, which are cell wall hydrolases and are produced late in the viral infection cycle, bind to peptidoglycan and disrupt the cell wall of Gram-positive bacteria that results in hypotonic lysis. Lysins have potential uses as antibacterials for human use. Systemic use of lysins has also been described (Fischetti *et al*., 2006), and may have advantages over whole bacteriophages because, in preclinical studies, they do not induce resistance, neutralizing antibodies or toxic shock. Currently, there is a lack of good human clinical trial results, although animal studies suggest that in certain circumstances, bacteriophage therapy may be useful.

6. The objectives of this study

As reviewed above, MRSA is the pathogen most frequently isolated worldwide from nosocomial infection. Vancomycin and teicoplanin have been used for the treatment of infections caused by MRSA, but resistant to vancomycin, vancomycin-intermediate and -resistant *S. aureus* (VISA and VRSA) have been reported in several countries. Recently, many substances against MRSA have been developed from organisms. However, little is known about the antibacterial activity of marine organisms against MRSA. Thus, the development of new drugs or alternative therapies is clearly a matter of urgency.

The objectives of this study, therefore, are to isolate and identify of anti-MRSA substances from marine organisms and to investigate the characteristics of the antibiotic active against MRSA.

Chapter 2. Materials and Methods

1. Bacterial strains and medium

The standard bacterial strains used in this study were purchased from the Korean Collection for Type Cultures (KCTC; Daejeon, Korea) or the Korea Culture Center of Microorganisms (KCCM; Seoul, Korea), respectively. Clinical MRSA isolates were generously provided by the Dong-A University Hospital (Busan, Korea). All strains were aerobically cultivated at 37°C in Mueller-Hinton broth (MHB; Difco, USA) for the minimum inhibitory concentration (MIC) assay, and in Mueller-Hinton agar (MHA; Difco, USA) for the disc diffusion assay.

2. Detection of *mecA* gene

In order to detect the *mec*A gene that encodes for a penicillin-binding protein, PBP2a, which is intrinsically insensitive to methicillin and to all β-lactams (Foster, 2004), MSSA and MRSA strain cells were cultured for 18 h at 37°C in Tryptic Soy Broth. Then, 3 ml of culture was collected and the chromosomal DNA was prepared using an *Accu-Prep* Genomic DNA Extraction Kit (Bioneer,

Daejeon, Korea). The chromosomal DNA was then used as a PCR reaction template. PCR was conducted using two synthetic oligonucleotides based on the results of current study (sense) 5'-AAAATCGATGGTAAAGGTTGGC-3' and (antisense) 5'-AGTTCTGC AGTACCGGATTTGC-3' (Murakami *et al.,* 2002). The DNA was amplified via 30 cycles of denaturation (94°C, 30 s), annealing (50°C, 30 s), and polymerization (72°C, 60 s). The amplified PCR product is anticipated to be approximately 500 bp.

3. Measurement of minimum inhibitory concentration (MIC)

The MICs were determined by a two-fold serial dilution method in MHB, as described by the National Committee for Clinical Laboratory Standards (2004). The MIC was defined as the lowest concentration of anti-MRSA substances that inhibited visual growth after 24 h of incubation at 37°C, and the assays were conducted in triplicates.

4. Isolation of anti-MRSA substances from *Ecklonia stolonifera*

4.1. Analytical methods

In order to identify an anti-MRSA substance from the solvent soluble fractions of *E. stolonifera,* a high performance liquid chromatography (HPLC) analysis was performed using a C_{18} reverse-phase column (ODS aqua, 125A, 250 × 10 mm, Phenomenix) with an Agilent 1100 HPLC system (Agilent Tech, Palo Alto, CA). For the detection of a bioactive substance, a linear gradient elution of 90% water with 10% methanol (v/v) to 100% methanol was used at a flow rate of 0.8 ml/min for 45 min. The eluate was monitored at 230 nm.

4.2. Isolation of dieckol

A lyophilized powder (3 kg) of *E. stolonifera* was percolated in hot ethanol (3 × 9L), followed by partitioning with several organic solvents to yield *n*-hexane (27.9 g), dichloromethane (25.6 g), ethyl acetate (25.0 g), and *n*-butanol (99.6 g) fractions (Fig. 4). The ethyl acetate fraction was then subjected to column chromatography over silica gel with ethyl acetate : methanol (50:1 to 5:1), yielding 10 subfractions (EF01 to EF10). The repeated column chromatography of

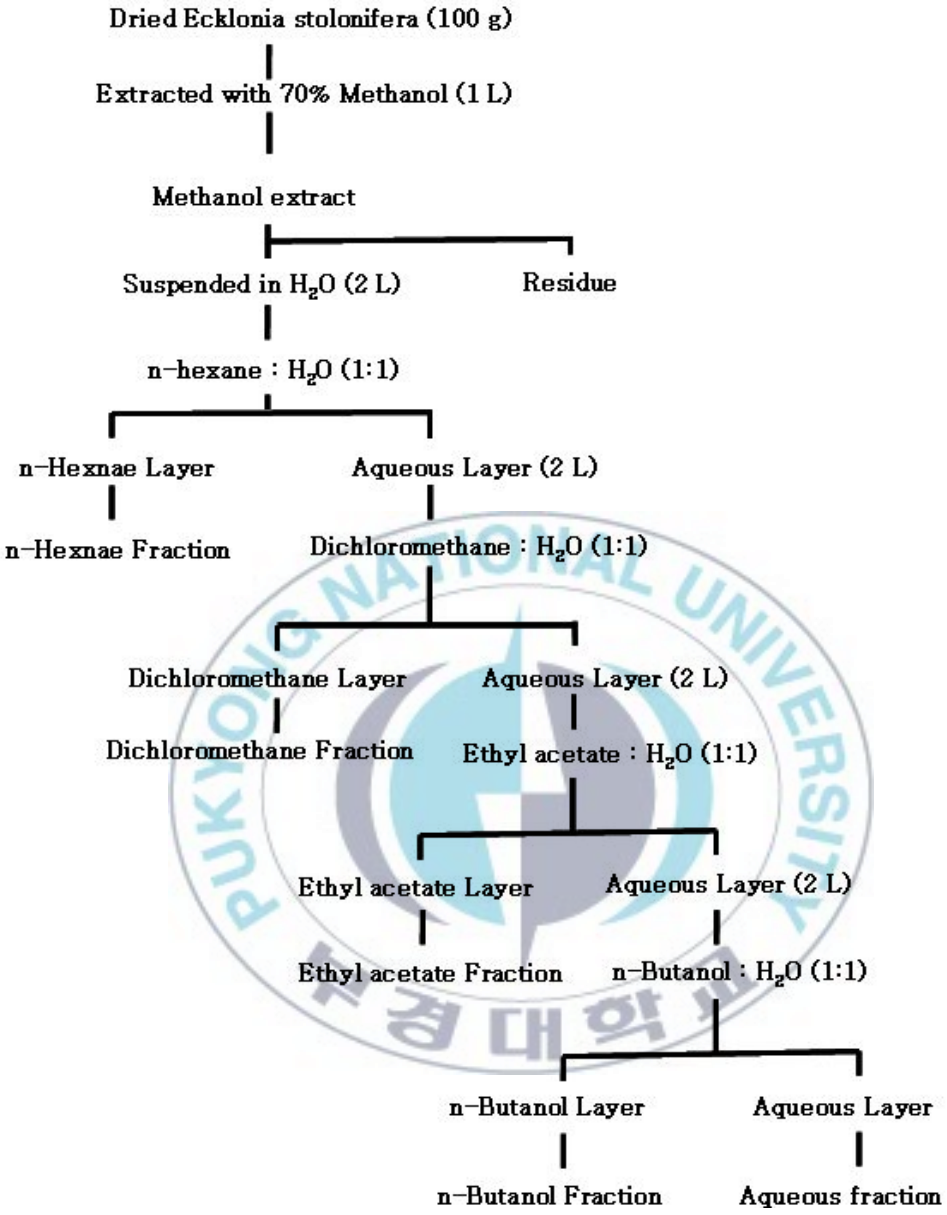


Fig. 4. Scheme of extraction and liquid-liquid solvent fraction.

fraction 1 (EF01, 3.44 g) was conducted with a solvent consisting of n-hexane and ethyl acetate, thereby yielding 11 subfractions (EF0101 to EF0111). Dieckol (87 mg) was purified from fraction 6 (EF0106, 945 mg), with RP-18 (20% methanol to 100% methanol, gradient) and Sephadex LH-20 (100% methanol).

The nuclear magnetic resonance (NMR) spectrum of dieckol is as follows. ^{1}H-NMR (400 MHz, CD_3OD) δ: 6.15 (1H, s, H-3"), 6.13 (1H, s, H-3), 6.09 (2H, s, H-2",6"), 6.06 (1H, d, *J* = 2.9 Hz, H-8), 6.05 (1H, d, *J* = 2.9 Hz, H-6"), 5.98 (1H, d, *J* = 2.8 Hz, H-6), 5.95 (1H, d, *J* = 2.8 Hz, H-6), 5.92 (3H, s, H-2′,4′,6′). ^{13}C-NMR (100 MHz, CD_3OD) δ: 162.7 (C-1′), 161.0 (C-3′,5′), 158.6 (C-1'"), 156.8 (C-7), 155.3 (C-7"), 153.2 (C-3'", 5'"), 148.1 (C-2"), 148.01 (C-2), 147.9 (C-9"), 147.7 (C-9), 145.1 (C-5a"), 145.0 (C-5a), 144.2 (C-4"), 144.1 (C-4'"), 139.4 (C-10a), 139.3 (C-10a"), 127.3 (C-4'"), 127.0 (C-9a), 126.5 (C-1), 126.4 (C-1"), 125.7 (C-9a"), 125.5 (C-4a"), 125.4 (C-4a), 100.7 (C-8"), 100.6 (C-8), 100.3 (C-3), 100.2 (C-3"), 98.5 (C-4′), 97.0 (C-2'",6'"), 96.7 (C-6"), 96.6 (C-6'), 96.2 (C-2′,6′). Positive FABMS *m/z* 742 $[M]^+$.

5. Synergistic effects between dieckol and β-lactams against MSSA and MRSA

The interaction between dieckol and β-lactams, including ampicillin, penicillin, and oxacillin, against MRSA were assessed by the checkerboard method (Norden *et al.*, 1979). The synergy between dieckol and the antibiotics was evaluated as a fractional inhibitory concentration (FIC) index (Norden *et al.*, 1979). The FIC was calculated as the MIC of an antibiotic or dieckol in combination, divided by the MIC of the antibiotic or dieckol alone, as follows. The FIC was then summed to derive the FIC index, which indicated synergy when the index values were: = 0.5, synergic; > 0.5 to = 1, additive; > 1 to = 2, independent and > 2, antagonistic.

FIC_A = MIC_A in combination / MIC_A,

FIC_B = MIC_B in combination / MIC_B

FIC Index = FIC_A + FIC_B

6. Isolation and characterization of anti-MRSA substances-producing microorganisms

6.1. Isolation of anti-MRSA substance-producing microorganisms

In order to isolate a bacterium producing anti-MRSA substance, sea water was collected and the samples were smeared on a PPES-II agar medium (0.2% polypeptone, 0.1% proteose peptone, 0.1% yeast extract, 0.1% soytone, 0.001% ferric citrate, and 2.5% NaCl; Taga, 1968) and incubated at 25℃ for 5 days. The anti-MRSA activity was estimated by a growth inhibition assay. MRSA were spread on Mueller-Hinton (MH) agar plate and a paper disk containing a cell-free culture broth of each isolated bacterium was then placed on the plate. After 2 days at 25℃, a strain, which made a clear zone indicating the MRSA growth inhibition, was selected.

6.2. Identification of anti-MRSA substance-producing microorganisms

The identification of the isolated strain was carried out by morphological observation, conventional biochemical tests, 16S rRNA gene sequence analysis and DNA–DNA hybridization. Morphological observation was carried out using a transmission electron microscope (JEM 1200EX-Ⅱ, JEOL, Tokyo, Japan) at the electron microscopy laboratory of Pusan Paik Hospital and gram staining. Biochemical

tests was carried out using the API 20 NE kit (bioMerieux, France).

Two oligonucleotides, based on the report of Dunbar *et al.* (2000), were used to determine 16S rDNA of the isolate: (Forward) 5'-AGAGTTTGATCCTGGCTCAG-3' and (Reverse) 5'-TACCTTGTTAC GACTT-3'. Polymerase chain reaction (PCR) was performed using intact cells, which were treated for 5 min at 95°C, as a template. The thermal profile was 25-cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 2 min at 72°C. A final extension step consisting of 5 min at 72°C was included. The 16S rDNA amplified by PCR was purified, cloned into pGEM-T easy vector (Promega, WI, USA), and sequenced. The analysis of nucleotide sequences was performed using the BLASTN (http://www.ncbi.nlm.nih.gov/BLAST) at the National Center for Biotechnology Information. Sequences were aligned using the program CLUSTAL W and a phylogenetic tree was made by using the MEGA 2.0 program.

6.3. Optimum culture conditions of *Pseudomonas* sp. UJ-6

Temperature, pH, and NaCl concentration was examined to determine the optimum culture condition in PPES-Ⅱ medium. For the temperature, cells were aerobically incubated in PPES-Ⅱ broth medium

at the following temperatures: 4, 15, 20, 25, 30, 37, and 50°C. The pH range for growth was determined by incubating cells in PPES-Ⅱ broth medium at the range of pH 4-10. Salt tolerance was tested on PPES-Ⅱ broth medium supplemented with NaCl 0-10% (w/v).

6.4. Anti-MRSA activity of *Pseudomonas* sp. UJ-6 culture

Pseudomonas sp. UJ-6 cells were pre-cultured at 25℃ in the PPES-Ⅱ broth. After overnight culture, the cells were diluted 50-fold into the fresh medium and the cell-free culture of UJ-6 was harvested at an indicated time. The culture was then concentrated ten fold using a rotary vacuum evaporator and the concentrated culture (160 μl/ml) was suspended into a MH broth inoculated by a MRSA strain (KCCM 40510), which was adjusted to an estimated cell density of about 10^4 CFU/ml. Then, the MRSA was cultured at 37℃ for 24 h. The cell growth of UJ-6 was monitored turbidimetrically at 640nm. The anti-MRSA activity was evaluated to determine viable cell counts of the MRSA strain after 24 h incubation.

7. Properties of the crude extract from *Pseudomonas* sp. UJ-6

7.1. Preparation of anti-MRSA substance from *Pseudomonas* sp. UJ-6 culture

The isolated UJ-6 cells were grown in PPES-Ⅱ broth medium (600L) at 25℃ for 48 h with agitating. The culture broth was centrifuged (15,000 × *g* at 4℃) and the supernatant was extracted with several organic solvents. Anti-MRSA activity of each fractions were tested and the active fraction, ethyl acetate fraction, was used for further study.

7.2. Effect of the crude extract on MRSA cell morphology

To compare the effects of the crude extract on MRSA cell morphology, MRSA cells were incubated at 37℃ for 24 h in presence (320 ㎍/ml) or absence of the extract. Then, a morphological change was observed using the TEM as descried above.

7.3. General characteristics of the crude extract

To investigate thermostability of the crude extract, the extract was incubated at several temperatures (4, 25, 50, 75, and 100℃) for 1 h. It was also autoclaved at 121℃ for 15 min. For the pH stability, the

crude extract was suspended in 0.1 M citrate phosphate buffer for the range of pH 3 to 7 and 0.1 M Tris-HCl buffer for pH 8 to 10, and then kept in each buffer for 30 min. After treatment, the anti-MRSA activity was estimated by the disk diffusion method. All assays were done in triplicate.

8. Isolation and structure elucidation of anti-MRSA compound in the culture broth of *Pseudomonas* sp. UJ-6

8.1. Instrumental analyses

^{1}H-, ^{13}C-, and 2D-NMR (nuclear magnetic resonance) spectra were recorded on a Varian Unity 500 NMR spectrometer in CD_3OD-d_4 at 300 K at Korea Ocean Research & Development Institute (KORDI). ^{1}H- and ^{13}C-NMR spectra were measured at 500 and 125 MHz, respectively. The resonances of residual CD_3OD-d_4 at δH 3.30 and δC 49.0 were used as internal references for ^{1}H- and ^{13}C-NMR spectra, respectively. The gHMBC spectra were acquired with a delay time of 60 ms. LC mass spectra, including high resolution mass measurements, were measured on the JEOL JMS-SX 102A mass spectrometer at KORDI. ^{1}H- and ^{13}C-NMR spectroscopic assignment analyses was performed using COSY, gHSQC, and gHMBC.

8.2. Isolations of anti-MRSA compound

The ethyl acetate (EtOAc) extract, which showed potent anti-MRSA activity, was concentrated by a rotary evaporator under vacuum at 40 °C. The residual suspension was subjected to ODS open vacuum flash chromatography with a stepwise gradient mixture of MeOH/H_2O as eluant followed by CH_2Cl_2. Then, the 80% MeOH fraction, which was anti-MRSA active fraction, subjected to normal-phase chromatography (silica gel chromatography) with a stepwise gradient mixture of hexane/EtOAc as eluant followed by MeOH. The fraction eluted with MeOH–H_2O (1:1, v/v) was purified by reversed-phase HPLC (YMC ODS-A column, 10 × 250 mm; 60% MeOH in H_2O; flow rate, 1.5 ml/min; RI detector). The procedure of purification and isolation of anti-MRSA compounds from *Pseudomonas* sp. UJ-6 is shown in Fig. 5.

9. Presentation of data

All experiments were conducted three or more times, and the data presented are from one of those experiments.

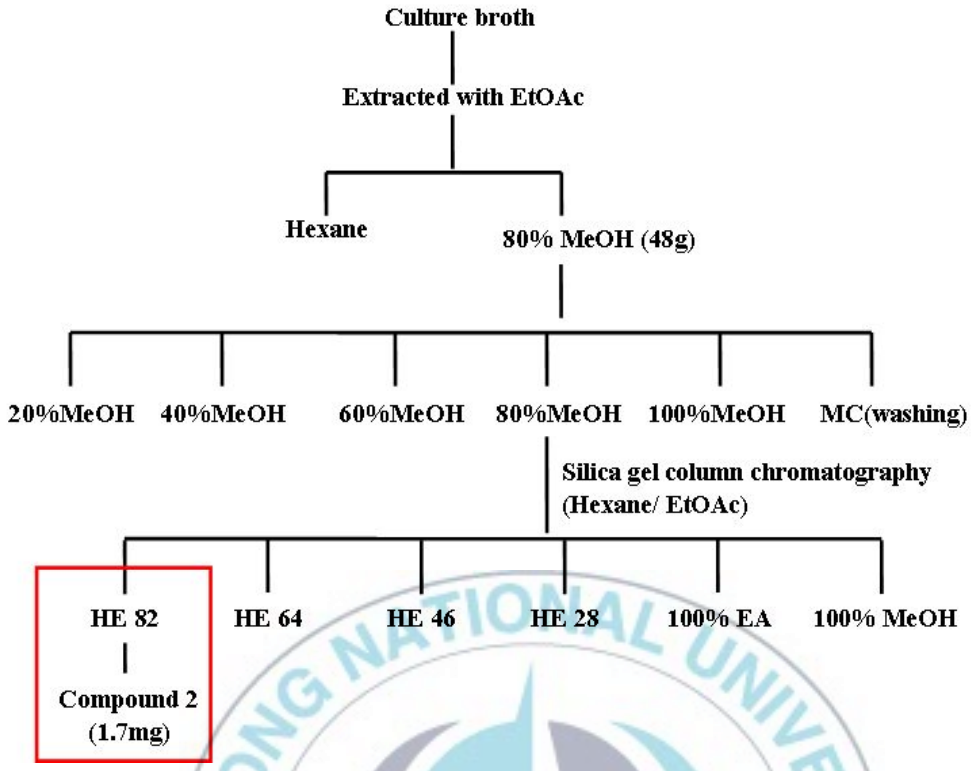


Fig. 5. Purification procedure of anti-MRSA compound from culture filtrate of *Pseudomonas* sp. UJ-6.

Chapter 3. Results and Discussion

Part 1. Isolation and characterization of anti-MRSA substances from *E. stolonifera*

1. Identification of an antibacterial substance against MRSA

In an effort to discover an alternative therapeutic agent against methicillin-resistant *Staphylococcus aureus* (MRSA), several medicinal plants and seaweeds were screened. Recently, it was reported that a methanolic extract of *E. stolonifera* exhibited significant antibacterial activity against MRSA (Eom *et al.*, 2008). Additionally, hexane and ethyl acetate soluble fractions from the methanolic extract evidenced a profound anti-MRSA activity, which was even more prominent than that of the methanol extract (Eom *et al.*, 2008). Other fractions, such as the dichloromethane and butanol fractions, demonstrated less pronounced anti-MRSA activity than was observed with the hexane and ethyl acetate fractions (Eom *et al.*, 2008).

In order to identify an active compound from the solvent soluble fractions, we conducted HPLC analyses (Fig. 6 and Fig. 7). The HPLC analyses showed that the methanolic extract and the ethyl acetate soluble fraction contained sizeable amounts of dieckol as a

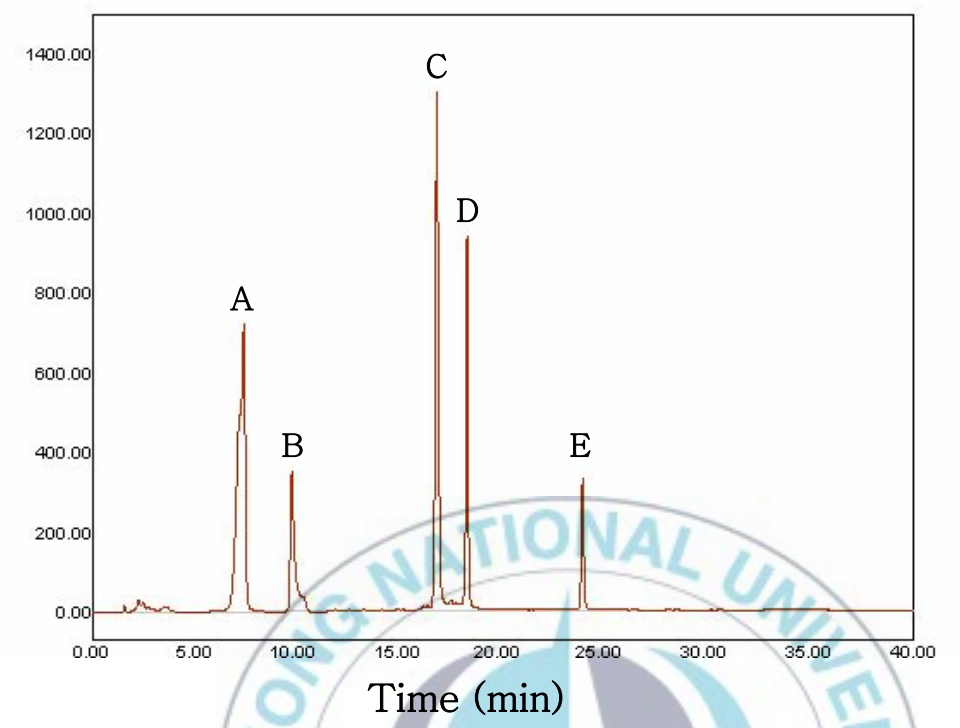


Fig. 6. HPLC elution profile of standard phlorotannins. Phlorotannins were isolated and analyzed by HPLC as described in Materials and Methods. A, phloroglucinol; B, triphlorethol-A; C, eckol; D, dieckol; E, eckstolonol.

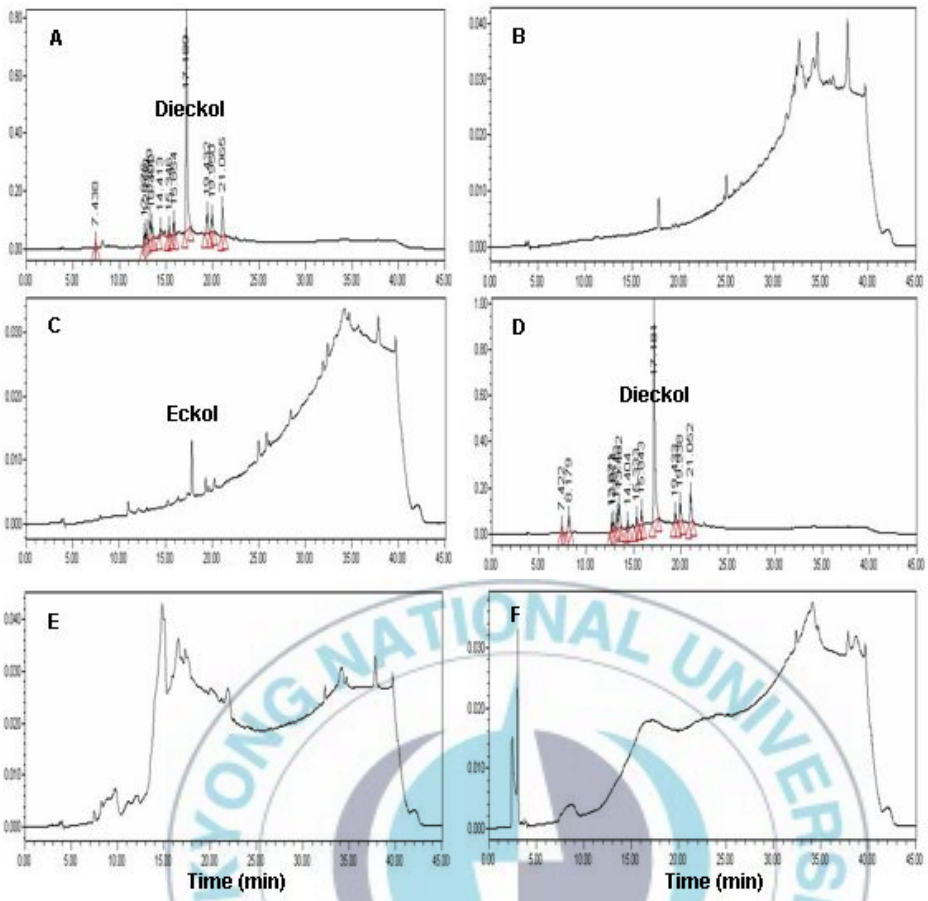


Fig. 7. HPLC elution profiles of the extracts of *Ecklonia stolonifera*. The HPLC conditions were the same as described in Fig. 6. A, methanolic extract; B, hexane soluble fraction; C, dichloromethane soluble fraction; D, ethyl acetate soluble fraction; E, butanol soluble fraction F, water soluble fraction.

major compound in relation to other phlorotannins (Fig. 8), all of which are polyphenols formed via the polymerization of phloroglucinol (1,3,5-trihydroxybenzene) units and are bio-active compounds known to function as antioxidant compounds (Taniguchi *et al.*, 1991). From these results, it was hypothesized that the anti-MRSA activity observed in the methanolic extract and the ethyl acetate fractions may have originated from dieckol. In order to test our hypothesis, we purified dieckol from *E. stolonifera* as described in the Materials and Methods section, and conducted a growth inhibitory assay against MRSA. As has been expected, the purified dieckol evidenced growth inhibitory activity against MRSA, thereby suggesting that dieckol is one of the anti-MRSA substances contained in *E. stolonifera*.

2. Antibacterial activity of dieckol

In order to quantitatively evaluate its antibacterial activity against MSSA and MRSA strains, the MIC value of dieckol was determined by a two-fold serial dilution method. Among the 13 strains tested in the present study, two standard MRSA and 10 clinical isolated strains were *mecA* positive, and one standard MSSA strain was found to be

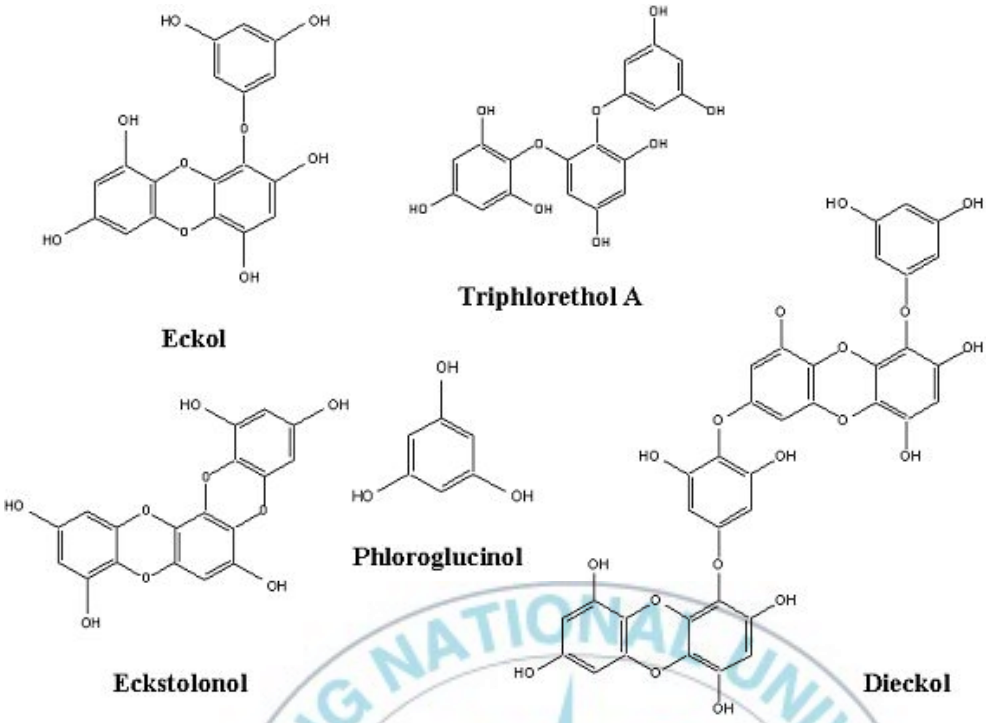


Fig. 8. Structure of phlorotannins isolated from *Ecklonia stolonifera.*

mecA-negative (Table 5). All of the MRSA strains were highly resistant to the β-lactams, including ampicillin, penicillin and oxacillin, and evidenced MICs equal to or greater than 64 ㎍/ml. The MICs of ampicillin, penicillin, and oxacillin against standard MSSA were less than 1 ㎍/ml, respectively, as has been established in other studies. The MICs of dieckol in the ranges of 32-128 ㎍/ml were equal to or less than those of the β-lactams tested against the MRSA strains (Table 5).

Dieckol evidenced a profound antibacterial activity against MSSA and MRSA, even though the β-lactams only displayed antibacterial activity against MSSA. It has been demonstrated that β-lactams inhibit several enzymes associated with the final step of peptidoglycan synthesis. Among them, ampicillin, penicillin, and oxacillin preferentially bind to PBP2 in the cell wall and inactivate their transpeptidase and carboxypeptidase activities these activities are responsible for catalyzing the final transpeptidation step of bacterial cell wall biosynthesis (Foster, 2004). The resistance of the β-lactam antibiotics, including ampicillin, penicillin, and oxacillin is mediated principally by the production of PBP2a encoded by the *mecA* gene (Foster, 2004). Owing to its low affinity for β-lactams, PBP2a provides transpeptidase activity to allow for cell wall synthesis at β -lactam concentrations that inhibit the β-lactam-sensitive PBP2 that is

Table 5. Minimum inhibitory concentration (MIC) of dieckol and β-lactams against methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA)

Strain	Source or reference	[b)]*mecA*	MIC (㎍ /ml)			
			Dieckol	Ampicillin	Penicillin	Oxacillin
MSSA (KCTC 1927)	Standard strain	-	32	< 0.125	< 0.125	< 0.125
MRSA (KCCM 40510)	Standard strain	+	64	512	512	128
MRSA (KCCM 40511)	Standard strain	+	64	512	256	128
MRSA D-3	Clinical isolate[a)]	+	64	128	128	512
MRSA D-4	Clinical isolate	+	128	128	256	128
MRSA D-6	Clinical isolate	+	64	256	128	256
MRSA D-8	Clinical isolate	+	64	256	128	512
MRSA D-11	Clinical isolate	+	64	128	128	64
MRSA D-12	Clinical isolate	+	64	128	128	64
MRSA D-13	Clinical isolate	+	128	128	256	256
MRSA D-14	Clinical isolate	+	64	128	64	128
MRSA D-17	Clinical isolate	+	64	128	128	128
MRSA D-18	Clinical isolate	+	128	128	128	512

[a)] MRSA strains were isolated in Dong-A University Medical Hospital

[b)] +, *mec*A positive; -, *mec*A negative

normally produced by MSSA. Considering the above, we supposed that the anti-MRSA activity of dieckol may not be related directly to PBP2a, as its antibacterial activity is not specific to MRSA.

It was also assessed the antibacterial activity of dieckol against the bacterial pathogens associated with food poisoning and spoilage (Table 6). Dieckol also evidenced antibacterial activity against the Gram-positive and -negative bacteria tested in the present study. The MICs of dieckol against bacterial pathogens were in the range of 64-256 μg/ml, thus indicating that this compound is less effective against Gram-negative bacteria than against Gram-positive bacteria.

It has been demonstrated that the modes of action of bacterial agents depend on the type of microorganisms, and are related primarily to their cell wall structure and to the arrangement of the outer membrane. The structure of the cell wall in gram-positive and –negative bacteria differs significantly. Considering this, it was surmised that these structural differences may be the main factor in the different susceptibilities to dieckol between gram-positive and gram-negative bacteria, and that the anti-MRSA activity of dieckol may be related principally to its direct or indirect effects on the bacterial cell wall.

Table 6. Antibacterial activity of dieckol against pathogenic bacteria.

	Strain	[c)]MIC (㎍/ml)	
		Dieckol	Vancomycin
Gram positive	[a)]MSSA (KCTC 1927)	32	0.5
	[b)]MRSA (KCCM40510)	64	2
	MRSA (KCCM 40511)	64	2
	Bacillus subtilis (KCTC 1028)	64	0.5
Gram negative	*Acinetobacter sp.* (KCTC 2011)	128	128
	Escherichia coli (KCTC 1682)	256	512
	Klebsiella pneumonia (KCTC 2001)	256	512
	Legionella birminghamensis (KCTC 2007)	256	256
	Salmonella typhimurium (KCTC 1925)	256	512
	Shigella flexneri (KCTC 2008)	256	256

[a)]Methicillin-susceptible *Staphylococcus aureus*, [b)]methicillin-resistant *S. aureus*, [c)]Minimum inhibitory concentration.

3. Synergic effects between dieckol and β-lactams against MSSA and MRSA

The β-lactam group of antibiotics includes an enormous variety of natural and semi-synthetic compounds, which inhibit several enzymes associated with the final step of peptidoglycan synthesis (Foster, 2004). All of these families are derived from a β-lactam structure. The multitude of chemical modifications based on this structure permits an astonishing array of antibacterial and pharmacological properties within this valuable family of antibiotics. The spread of drug-resistance bacteria and the ever-increasing development of pathogenic microbial resistance to traditional antibiotics has already reached alarming levels thus the development of new drugs or alternative therapies is clearly a matter of urgent necessity (Hiramatsu *et al.*, 1997; Hanaki *et al.*, 1998). It has been demonstrated that one of the more effective strategies in this regard is the restoration of antibiotic activity in combination with antibacterial materials derived from natural products and traditional medicines against drug-resistance bacteria (Zaho *et al.*, 2001; Shioda *et al.*, 2004; Jeong *et al.*, 2006). Thus, it was assessed the synergistic effects of dieckol on MRSA when administered in combination with commercial β-lactams.

As is shown in Table 7, the MICs of ampicillin against two standard MRSA strains (KCCM 40510 and 40511) were reduced dramatically, from 512 to 0.5 ㎍/ml when administered in combination with 16 ㎍/ml of dieckol, thereby indicating that dieckol reversed the high-level ampicillin resistance of MRSA. The synergistic effect against MRSA was also verified in combination with dieckol and penicillin. The synergy was evaluated in terms of a FIC index between dieckol and β-lactams, as described in the Materials and Methods section. As summarized in Table 7, the FIC indices of ampicillin were in a range from 0.133 to 0.254 in combination with a low concentration of dieckol (16 ㎍/ml) against two standard and 10 isolates of MRSA, thereby indicating the synergistic effect of the dieckol-ampicillin combination. The MICs of penicillin against MRSA strains were also reduced when administered in combination with dieckol. The FIC indices of penicillin occurred in a range from 0.127to 0.258 in combination with 16 ㎍/ml of dieckol against all tested MRSA strains, thereby indicating that dieckol and penicillin synergistically inhibit the growth of MRSA. However, no synergy was observed between dieckol and oxacillin against two standard and 6 isolates of MRSA strains. Differences in the synergistic effects of an anti-MRSA substance-β-lactams combination was also reported in

Table 7. Minimum inhibitory concentrations (MICs) and fractional inhibitory concentration (FIC) indices of dieckol in combination with β-lactams against methicillin-resistant *Staphylococcus aureus* (MRSA)

Strain	Ampicillin					Penicillin					Oxacillin				
	MIC (㎍/㎖)			FIC index		MIC (㎍/㎖)			FIC index		MIC (㎍/㎖)			FIC index	
	A	B	C	b	c	A	B	C	b	c	A	B	C	b	c
MRSA40510	512	1	0.5	0.127	0.251	512	2	1	0.129	0.252	128	128	128	1.125	1.250
MRSA40511	512	1	0.5	0.127	0.251	256	1	0.5	0.129	0.252	128	128	128	1.125	1.250
MRSA[a)] D-3	128	1	0.5	0.133	0.254	128	2	1	0.141	0.258	512	512	512	0.375	0.258
MRSA D-4	128	2	1	0.078	0.133	256	1	0.5	0.066	0.127	128	8	4	0.125	0.188
MRSA D-6	256	1	0.5	0.129	0.252	128	1	0.5	0.133	0.254	256	16	8	0.188	0.313
MRSA D-8	256	1	0.5	0.129	0.252	128	2	1	0.141	0.258	512	32	16	0.188	0.500
MRSA D-11	128	1	0.5	0.133	0.254	128	2	1	0.141	0.258	64	64	64	1.125	1.250
MRSA D-12	128	1	0.5	0.133	0.254	128	2	1	0.141	0.258	64	64	64	1.125	1.250
MRSA D-13	128	2	1	0.078	0.133	256	1	0.5	0.066	0.127	256	16	8	0.125	0.156
MRSA D-14	128	1	0.5	0.133	0.254	64	2	1	0.156	0.266	128	128	128	1.125	1.250
MRSA D-17	128	1	0.5	0.133	0.254	128	1	0.5	0.133	0.254	128	128	128	1.125	1.250
MRSA D-18	128	2	1	0.078	0.133	128	1	0.5	0.070	0.129	512	512	512	0.563	0.625

A, without dieckol; B to C and b to c, dieckol at 8 and 16 μg/ml, respectively. [a)] The FIC index indicated synergy: = 0.5, synergic; > 0.5 to = 1, additive; > 1 to = 2, independent, > 2, antagonistic.

epigallocatechin gallate (EGCg). EGCg evidenced synergy against MRSA in combination with penicillin and oxacillin, but this synergy was not observed with an EGCg-ampicillin combination (Zaho *et al.*, 2001). This discrepancy in susceptibilities may be attributable to structural differences between dieckol and EGCg. Dieckol is a hexamer of phloroglucinol; thus, it has a rather bulkier structure than EGCg. It appears that dieckol (or EGCg) and penicillin synergistically attack the same site of the cell wall. However, no synergistic binding between dieckol and oxacillin will occur at the target site(s), as has been reported previously in between EGCg and β-lactams (Zaho *et al.*, 2001).

Dieckol reversed the high-level resistance of MRSA to ampicillin and penicillin but not to oxacillin, and the FIC indices between dieckol and the two antibiotics (ampicillin and penicillin) also differed, thereby implying that the anti-MRSA mechanism between β-lactams in combination with dieckol may differ. Additionally, dieckol is less effective against Gram-negative bacteria than against Gram-positive bacteria as is shown in Table 6. Considering the above results, it is hypothesized that dieckol may synergize the activity of β-lactams, which inhibit cell wall synthesis, due to the inhibition of the function or synthesis of the bacterial cell wall, even though further experiments

will be required in order to verify the hypothesis.

As mentioned above, MRSA is most problematic gram-positive bacterium, due to its resistance to almost all available antibiotics, with the exception of vancomycin (Witte, 1999; Isnansetyo and Kamei, 2003). As the application of vancomycin has become more widespread, the susceptibility of MRSA to vancomycin has decreased in recent years. Therefore, there is currently a pressing need to develop new drugs or alternative therapies (Hiramatsu *et al.*, 1997; Hanaki *et al.*, 1998). As is shown in Tables 5 and Table 6, the MICs of ampicillin and penicillin against MRSA were determined to range between 0.5 and 1 μg/ml when administered in combination with 16 μg/ml of dieckol. This value is less than that measured for vancomycin (2 μg/ml), which suggests that dieckol may have potential for use as an adjunct in the treatment of antibiotic-resistant bacteria.

The results of the present study are expected to contribute to the development of an alternative phytotherapeutic agent against MRSA, and in applications of the treatment of MRSA infections. Moreover, this attractive brown alga, *E. stolonifera,* and its component, dieckol, appear to harbor some potential as promising pharmaceuticals, including both anti-MRSA substances and bioactive nutraceuticals.

Part 2. Isolation and characterization of anti-MRSA substances from marine bacterium, *Pseudomonas* sp. UJ-6

1. Isolation of an anti-MRSA substance producing microorganism

A bacterial strain UJ-6, which exhibits an anti-MRSA activity, was isolated from sea water collected in Uljin, Gyungbuk Province, Korea (Fig. 9). The cell-free culture of the UJ-6 was able to a clear zone against MRSA, indicating that the UJ-6 produces an anti-MRSA substance.

2. Identification of an anti-MRSA substance-producing microorganism

In order to identify this strain, morphological, biochemical and genitival analyses were performed. Gram staining reveals that this strain is Gram negative rod (Fig. 10) and the color of colony was creamy-yellow on PPES-Ⅱ agar plate. Transmission electron micrograph (TEM) indicates that the UJ-6 strain is rod-shaped non-spore forming cell with rounded end and is approximately 1.0 μm long and 0.4 μm in diameter (Fig.11). The results of biochemical tests were listed in Table 8. The strain utilized glucose, arabinose,

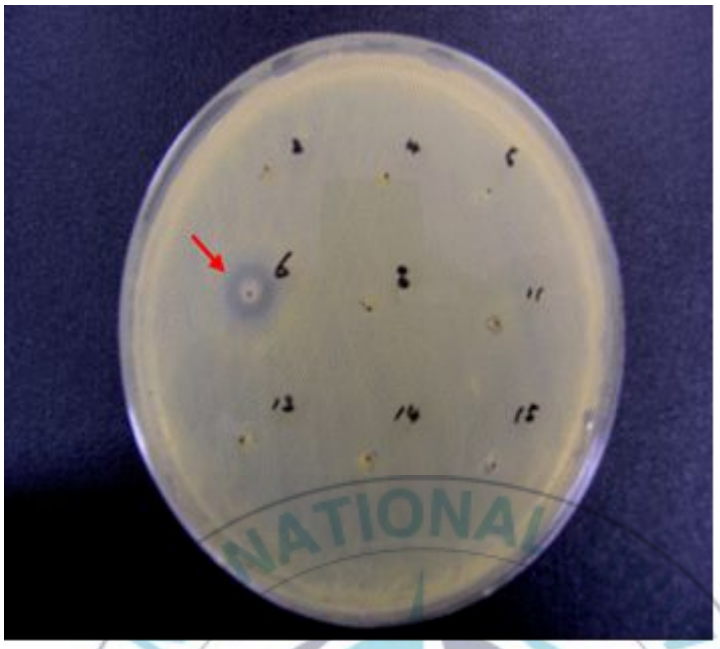


Fig. 9. Isolation of an anti-MRSA substance producing microorganism. Arrow bar indicated the isolated strain UJ-6.

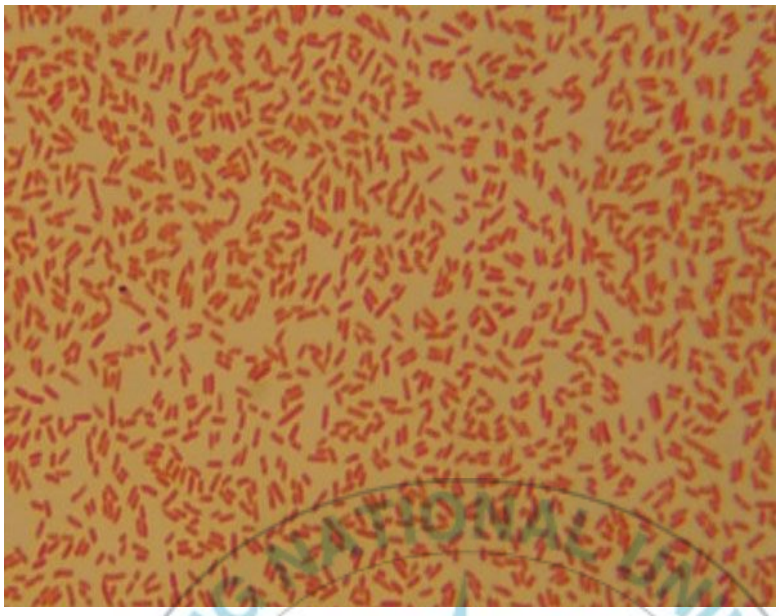


Fig. 10. Gram staining of *Pseudomonas* sp. UJ-6.

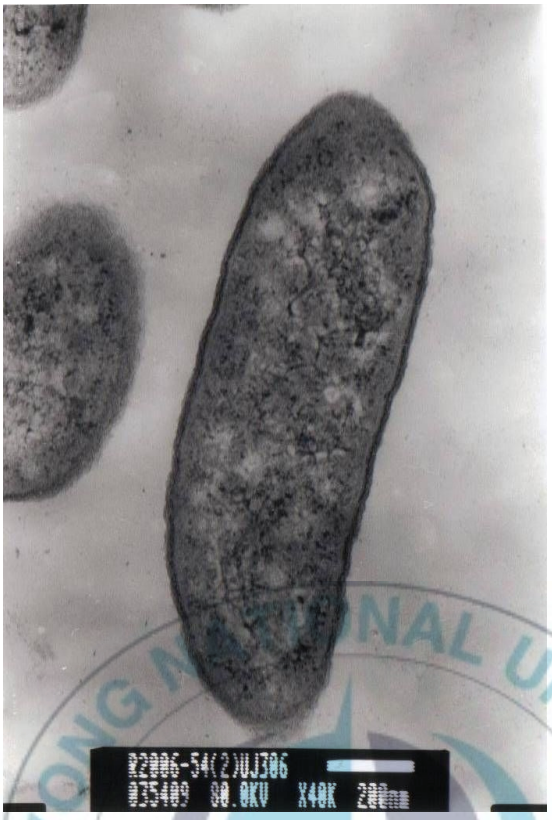


Fig. 11. Transmission electron micrograph of *Pseudomonas* sp. UJ-6. Scale bar represents 200 nm in size.

Table 8. Biochemical characteristics of *Pseudomonas* sp. UJ-6 and type strains belonging to the genus *Pseudomonas*

	1	2	3	4	5
Reduction of nitrates to nitrites	-	-	-	+	+
Indole production	-	-	-	-	-
Glucose acidfication	-	-	-	-	-
Arginine dehydrolase	+	+	-	-	-
Urease	-	-	-	-	-
Esculin hydrolysis (β-glucosidase)	-	-	-	-	-
Gelatine hydrolysis (protease)	+	-	-	-	-
β-galactosidase	-	-	-	-	-
Glucose assimilation	+	+	+	+	+
Arabinose assimilation	+	+	-	+	+
Mannose assimilation	+	+	+	+	+
Mannitol assimilation	+	+	+	+	+
N-acetyl-glucosamine assimilation	+	+	+	+	+
Maltose assimilation	-	-	-	-	-
Gluconate assimilation	+	+	+	+	+
Caprate assimilation	+	+	+	+	+
Adipate assimilation	-	-	+	-	+
Malate assimilation	+	+	+	+	+
Citrate assimilation	+	+	+	+	+
Phenyl-acetate assimilation	-	-	-	-	-
Cytochrome oxidase	+	+	+	+	+

The taxa are 1, *Pseudomonas* sp. UJ-6; 2, *P. reactans* (LMG 5329) 3, *P. gessardii* (CIP 105469); 4, *P. synxantha* (IAM 12356); 5, *P. azotoformans* (IAM 1603). +, positive result or growth; -, negative result or no growth

mannose, mannitol, *N*-acetyl-glucosamine, gluconate, caprate, malate, and citrate but did not assimilate maltose, adipate, and phenyl-acetate. Cytochrome oxidase, gelatine hydrolysis, and arginine dehydrolase were positive.

For genetic characterization of strain UJ-6, PCR was carried out to amplify 16S rDNA as described above. The 16S rDNA sequences (1457 bases) of strain UJ-6 was aligned by comparison with available sequences from GenBank. The sequences of strain UJ-6 shared the greatest similarity with those of *P. reactans* LMG 5329 and *P. gessardii* CIP 105469 (99.7 and 99.3% similarity, respectively). A phylogenetic tree based on bacterial 16S rDNA sequences showed close relationships of UJ-6 with the genera *Pseudomonas* (Fig. 12).

The isolated strain was identified to be *Pseudomonas* sp. by the morphology, biochemical reactions, and homology research based on 16S rDNA sequencing analysis. Based on these results, the isolated UJ-6 was identified to be *Pseudomonas* sp..

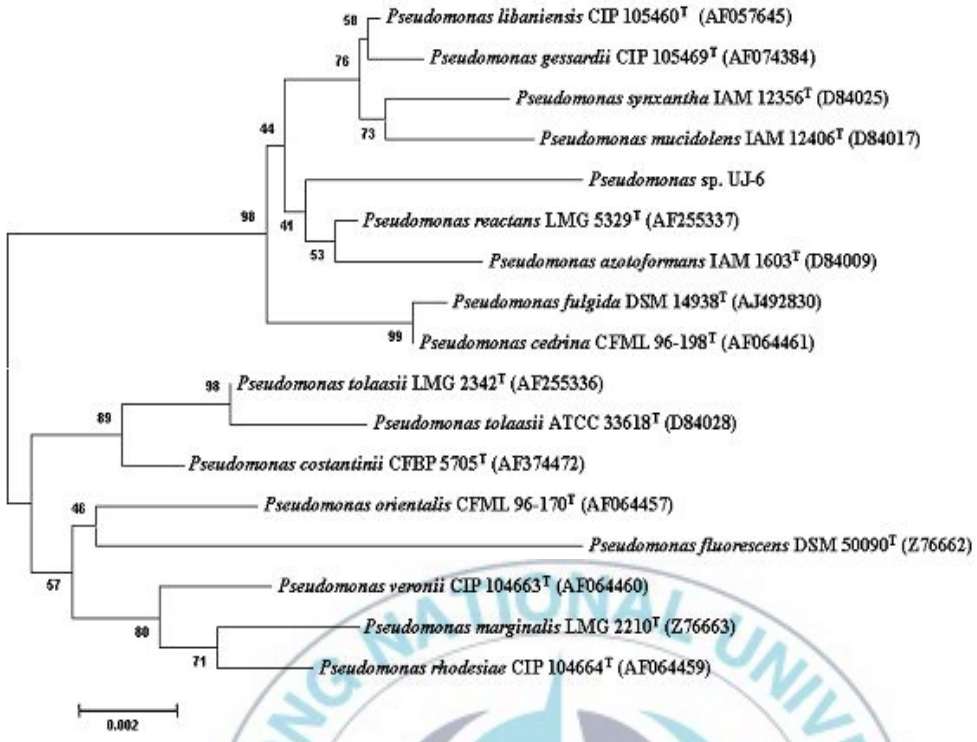


Fig. 12. Phylogenetic tree based on 16S rDNA sequences of *Pseudomonas* sp. UJ-6 and closely related members of the genus *Pseudomonas*. Number at nodes are levels of bootstrap support based on neighbour-joining analyses of 1000 replications.

3. Optimum culture conditions of *Pseudomonas* sp. UJ-6

To determine optimum culture conditions of *Pseudomonas* sp. UJ-6, the cell was incubated at different temperatures, pH, and NaCl concentration. *Pseudomonas* sp. UJ-6 is able to grow in ranging from 4℃ to 37℃ but not over at 50℃ (Fig. 13). Also, the strain grows well between pH 5.0 and 9.0 but the growth is inhibited below pH 4.0 and above pH 10.0 (Fig. 14). High concentration of NaCl (over 5%) resulted in the retardation of growth or no growth (over 9% NaCl). Thus, the most favorable growth of *Pseudomonas* sp. UJ-6 is observed in the PPES-Ⅱ medium containing 1% NaCl, adjusted to pH 7.0, and incubated at 25℃ (Fig. 15). However, there was no significant difference in anti-MRSA activity between different culture conditions.

4. Anti-MRSA activity of *Pseudomonas* sp. UJ-6

Pseudomonas sp. UJ-6 showed the anti-MRSA activity as shown in Fig. 9. It was further investigated whether the strain UJ-6 produces

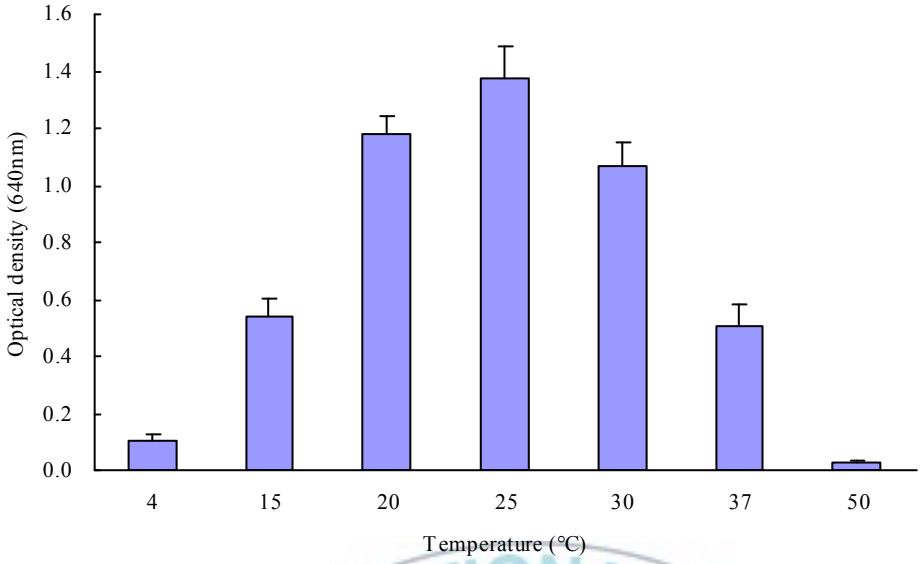


Fig. 13. Effects of temperature on the growth of *Pseudomonas* sp. UJ-6 in PPES-II medium. For the temperature, cells were aerobically incubated in PPES-Ⅱ broth medium (pH 7, 2% NaCl) at the following temperatures: 4, 15, 20, 25, 30, 37, and 50°C.

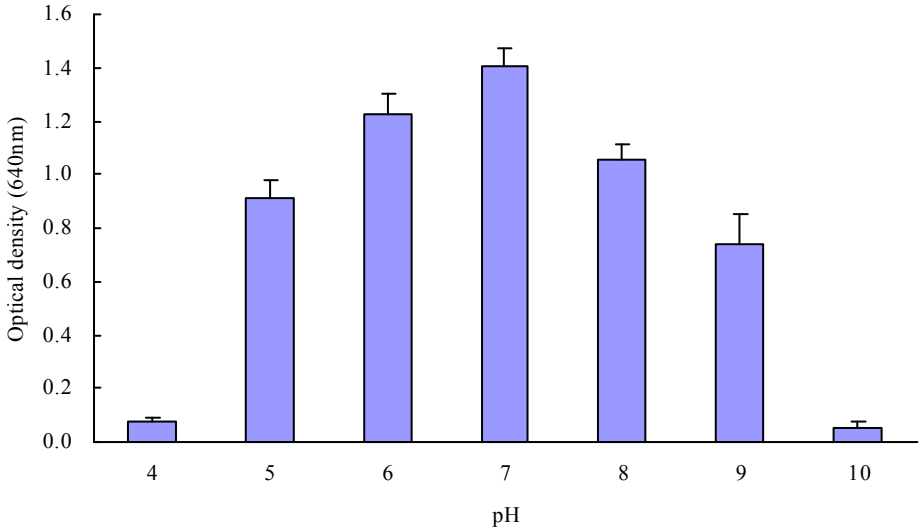


Fig. 14. Effects of pH on the growth of *Pseudomonas* sp. UJ-6 in PPES-II medium. The pH range for growth was determined by incubating cells in PPES-Ⅱ broth medium (2% NaCl, 25°C) at the range of pH 4-10.

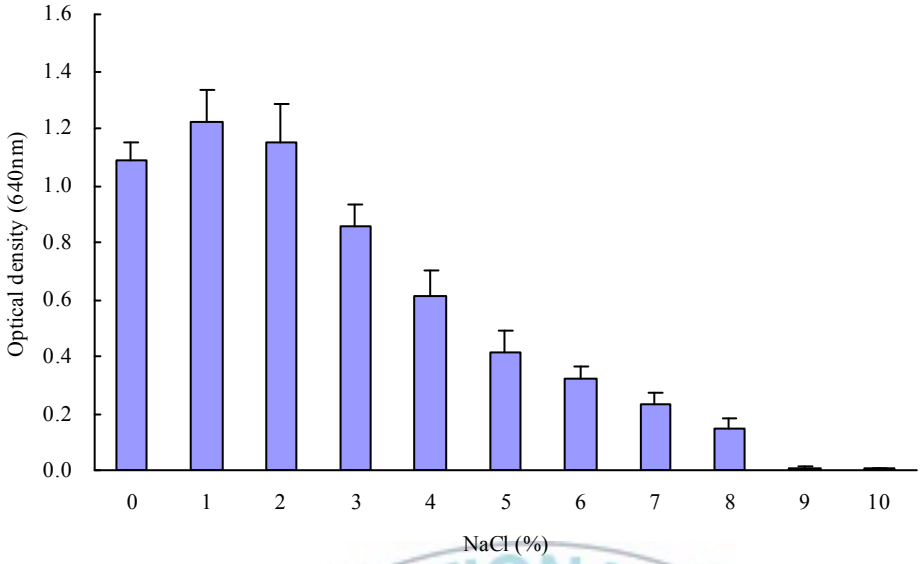


Fig. 15. Effects of NaCl concentration on the growth of *Pseudomonas* sp. UJ-6 in PPES-II medium. Salt tolerance was tested on PPES-Ⅱ broth medium (pH 7, 25°C) supplemented with 0-10% (w/v).

bacteriostatic or bactericidal substance. The culture of *Pseudomonas* sp. UJ-6 showed a bactericidal activity against MRSA, indicating that the UJ-6 produces a bacterial substance (Fig. 16). The strongest activity was observed after stationary phase of growth (Fig. 16). In order to elucidate the anti-MRSA mechanism and purify an active compound from the culture of strain UJ-6, the culture was extracted with several organic solvents such as ether, hexane, chloroform, methylene chloride, and ethyl acetate. Among them, the ethyl acetate extract only showed significant an antibacterial activity against all the tested gram-positive species including MRSA strains and all the tested gram-negative species. MICs of the ethyl acetate extract against MRSA strains and other bacteria were shown in Table 9. The ethyl acetate extract showed an antibacterial activity against MRSA strains tested with MICs ranging from 160 to 320 μg/ml. The extract also exhibited an antibacterial activity against Gram-negative bacteria, even though the extract was less effective against Gram-negative bacteria and *S. iniae* than against other Gram-positive bacteria. However, vancomycin was not effective against Gram-negative bacteria, suggesting the anti-MRSA mechanism by the substance produced by UJ-6 will differ from that of vancomycin. These results are similar with the result of other marine bacterium producing an anti-MRSA substance (Isnansetyo and Kamei, 2003).

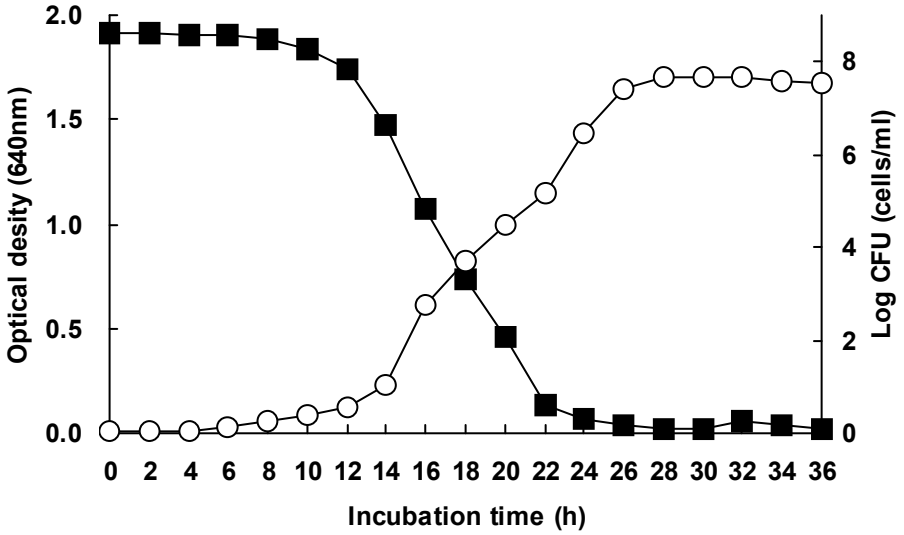


Fig. 16. Relationship between cell growth of *Pseudomonas* sp. UJ-6 and anti-MRSA activity (○, *Pseudomonas* sp. UJ-6; ■, MRSA, methicillin-resistant *Staphylococcus aureus*). *Pseudomonas* sp. UJ-6 cells were grown in the PPES-II broth at 25℃ and the cell-free culture broth of UJ-6 was harvested at an indicated time. Then, ten folds concentrated broth (160 μl/ml) was added into a Mueller-Hinton broth inoculated by a MRSA strain (KCCM 40510), which was adjusted to an estimated cell density of about 10^4 CFU /ml. Then, the MRSA was cultured at 37℃ for 24 h. The cell growth of UJ-6 was monitored turbidometrically at 640nm. The anti-MRSA activity was evaluated to determine viable cell counts of the MRSA strain after 24 h incubation.

Table 9. Antibacterial activity of the ethyl acetate extract of *Pseudomonas* sp. UJ-6 culture

Strains	[a]MIC (μg/ml)	
	Ethyl acetate extract	vancomycin
[b]MRSA (KCCM 40510)	320	2
MRSA (KCCM 40511)	320	2
MRSA (DH 70503)	320	2
MRSA (DH 70504)	320	2
MRSA (DH 70505)	320	1
MRSA (DH 70506)	160	2
MRSA (DH 70508)	640	2
MRSA (DH 70510)	320	1
MRSA (DH 70512)	640	2
MRSA (DH 70513)	640	1
MRSA (DH 70514)	640	2
MRSA (DH 70517)	320	1
MRSA (DH 70518)	320	2
MRSA (DH 70519)	640	2
Staphylococcus aureus (KCTC 1621)	320	1
Staphylococcus aureus (KCTC 1927)	160	0.5
Staphylococcus aureus (KCTC 1928)	320	1
Bacillus subtilis (KCTC 1028)	160	0.5
Streptococcus iniae (KCTC 3657)	640	4
Escherichia coli (KCTC 1682)	640	512
Pseudomonas aeruginosa (KCTC 1637)	640	512
Salmonella typhimurium (KCTC 1925)	640	512
Vibrio parahaemolyticus (KCTC 2729)	1280	512
Klebsiella pneumoniae (KCTC 2001)	640	512

Pseudomonas sp. UJ-6 was cultured and extracted as described in Material and methods. [a]MIC (Minimum inhibitory concentration) of crude extract and vancomycin was determined by the two-fold serial dilution method in Mueller-Hinton broth. [b]MRSA, methicillin-resistant *Staphylococcus aureus*.

5. Effect of the ethyl acetate extract on MRSA cell morphology

It was also investigated a morphological change of MRSA cell by the ethyl acetate extract using a transmission electron microscopy. As shown in Fig. 17, a lysis of MRSA cell has been observed when the cell was grown at 37℃ for 24 h with the ethyl acetate extract (320 ㎍/ml). It is well known that several antibiotics interfere with cell wall synthesis, as do penicillin and vancomycin, eventually leading to lysis of cell (Barna and Williams, 1984). From these results, it was supposed that *Pseudomonas* sp. UJ-6 also produces a substance to interfere with cell wall synthesis of MRSA. However, we strongly believe that the anti-MRSA mechanism by *Pseudomonas* sp. UJ-6 differs from that of vancomycin since vancomycin was not effective against Gram-negative bacteria.

6. Thermal and pH stability of the ethyl acetate extract

Thermal and pH stability of the ethyl acetate extract was investigated. The extract kept over 95% activity at pH 3.0–8.0 but exhibited about 80% and 60% activity at pH 9.0 and 10.0, respectively,

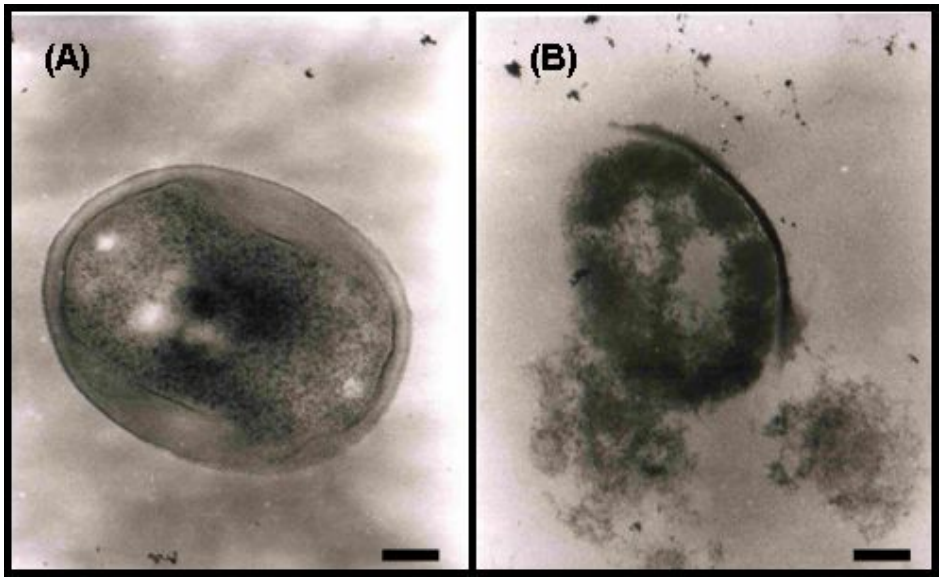


Fig. 17. Abnormal cell morphology of MRSA caused by the ethyl acetate extract of *Pseudomonas* sp. UJ-6 culture. A MRSA strain (KCCM 40510, 10^5 CFU/ml) was inoculated in a Mueller-Hinton broth h in the absence or presence of the ethyl acetate extract (320 μg/ml). The culture was incubated at 37℃ for 24 h and the cell morphology was observed with a transmission electron microscopy. (A) Normal cell of the MRSA (B) abnormal cell lysis of the MRSA grown with the ethyl acetate extract. The scale bar indicates 100 nm.

when the activity at pH 7.0 was defined as 100% (Fig. 18 A). As shown in Fig. 18, the extract was very stable to thermal stress. The extract kept over 95% of its activity after heat treatment for 15 min at 121℃ (Fig. 3-18 B). This result suggests that *Pseudomonas* sp. UJ-6 produces a thermal stable antibiotic, even though most known antibiotics are unstable to thermal stress. To address these issues, it will be necessary to confirm the structure of anti-MRSA compound from the crude extract. From these results, it is anticipated that *Pseudomonas* sp. UJ-6 will provide valuable insights to develop a novel antibiotic against the MRSA due to its thermal stability and its broad ranging antibacterial activity.

7. Isolation of anti-MRSA compound

In the process of solvent extraction, the anti-MRSA activity was detected in the ethyl acetate extract (48 g). The ethyl acetate extract was concentrated and the residual aqueous suspension was subjected to ODS vacuum flash chromatography with aqueous MeOH followed by methylene chloride. The active fraction against MRSA is observed

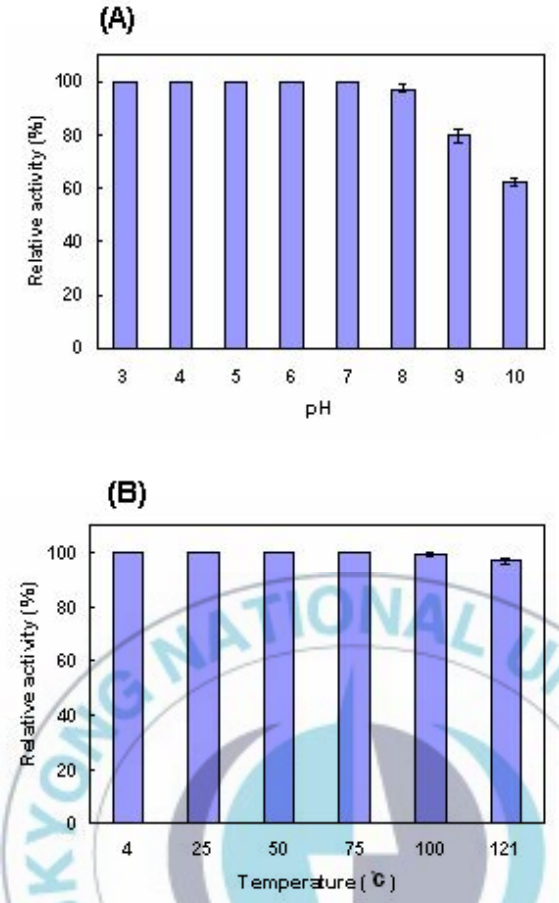


Fig. 18. The pH (A) and thermal stability (B) of the ethyl acetate extract of *Pseudomonas* sp. UJ-6 culture. For pH stability, the extract was suspended in 0.1 M citrate phosphate buffer for the range of pH 3 to 7 and 0.1 M Tris-HCl buffer for pH 8 to 10, and then kept in each buffer for 30 min. For thermal stability, the extract was incubated at an indicated temperature (4, 25, 50, 75, and 100℃) for 1 hr or at 121℃ for 15 min. After treatment, the anti-MRSA (methicillin-resistant *Staphylococcus aureus*) activity was estimated by the disk diffusion method. All assays were done in triplicate.

in 80% MeOH fractions (Fig. 19). Then, the active fraction was subjected to silica gel chromatography with a stepwise gradient mixture of hexane /EtOAc as eluant followed by MeOH. After then, active fractions are observed in hexane/EtOAc (8:2) fraction (70 ㎎), EtOAc 100% fraction (540 ㎎) and 100% MeOH fraction (360 ㎎) (Fig. 20). The final purification was achieved by reversed-phase HPLC as described in Materials and Methods. It was obtained 2 pure compounds, compound 2 (1.7 ㎎) and compound 9 (1.3 ㎎), exhibiting anti-MRSA activity from hexane/EtOAc (8:2) fraction (Fig. 21). These compounds were subjected to structural analysis.

8. Structure determination of anti-MRSA compound 2

Structure determination of anti-MRSA compound was performed using ^{1}H-, ^{13}C-, and 2D-NMR and LC-Mass analyses. ^{1}H- and ^{13}C-NMR spectroscopic assignment analyses was performed using COSY, gHSQC, and gHMBC. LC-mass spectrum of anti-MRSA compound indicated that molecular weight of compound 2 is 210.07 (Fig. 22) and depicted as **1** (Fig. 23). In the mass spectrum, CO peak at *m/z* 182 and $[(M - (COCH_2)]^+$ base peak at *m/z* 168 also established the

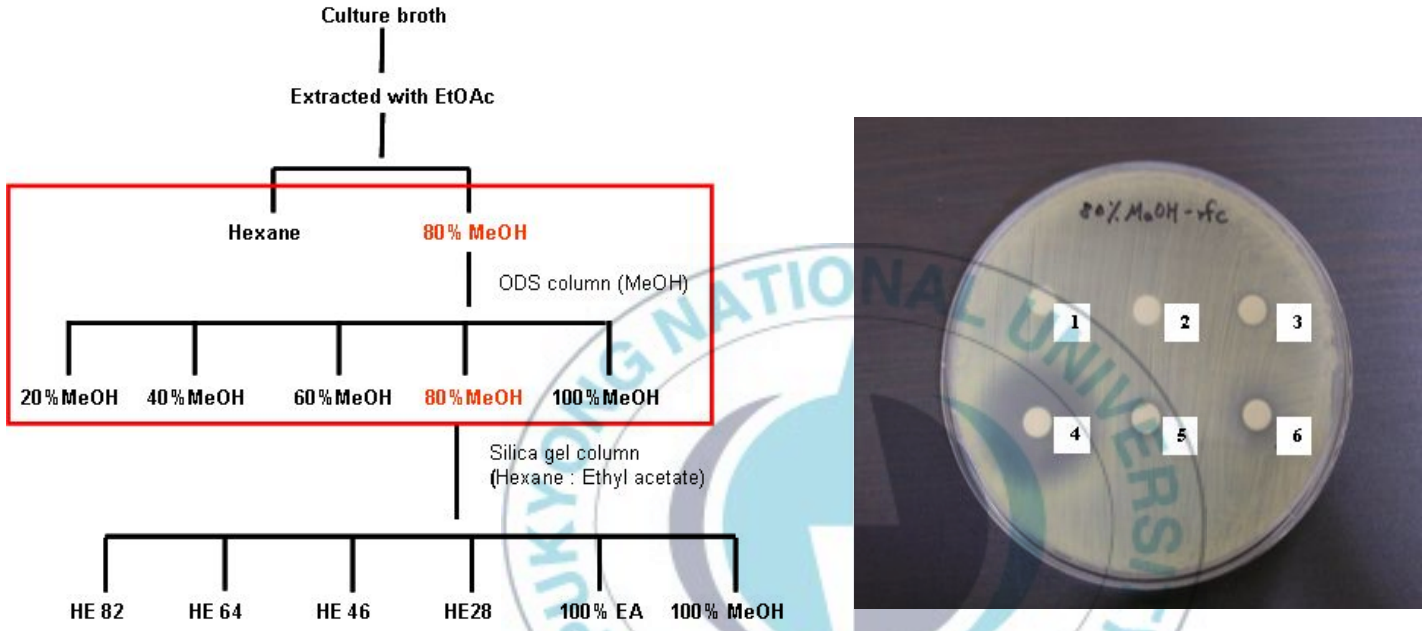


Fig. 19. The anti-MRSA activity of ODS vacuum flash column fractions. The ethyl acetate extract was subjected to ODS vacuum flash chromatography with aqueous MeOH followed by CH_2Cl_2.
1, 20% MeOH; 2, 40% MeOH; 3, 60% MeOH; 4, 80% MeOH; 5, 100% MeOH 6, 100% CH_2Cl_2.

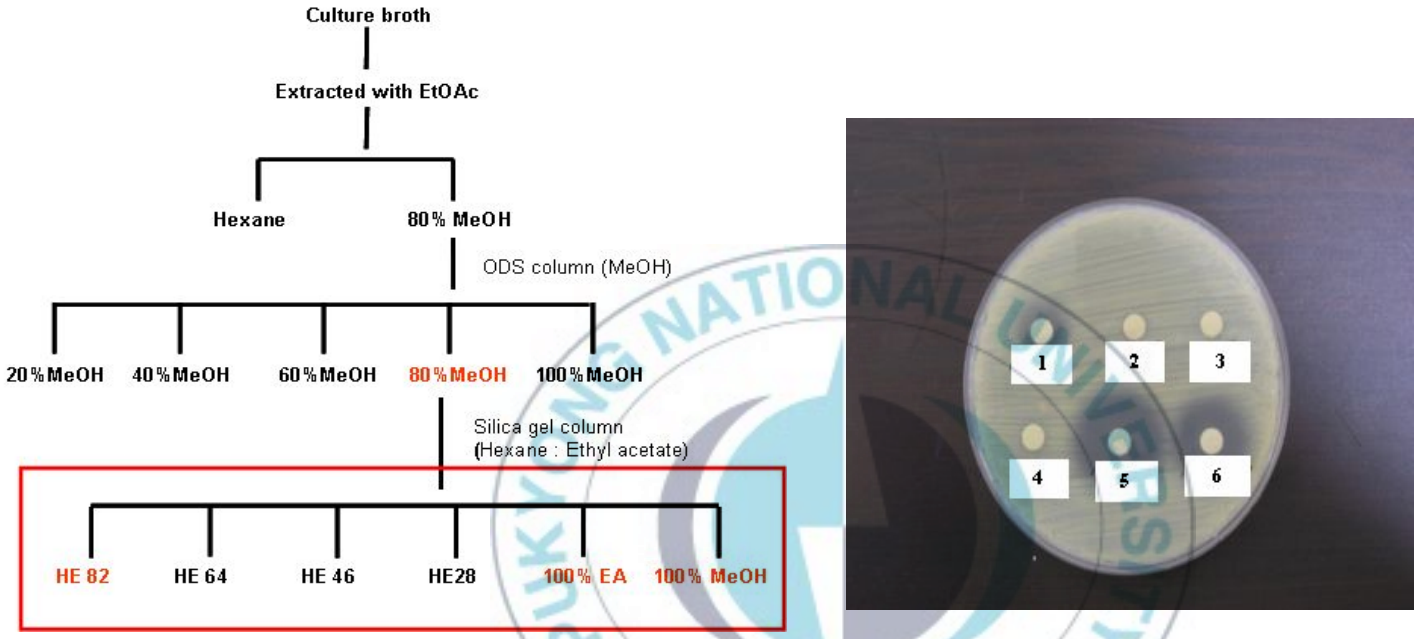


Fig. 20. The anti-MRSA activity of silica gel column fractions. The 80% MeOH fraction was subjected to silica gel chromatography with a stepwise gradient mixture of hexane/EtOAc as eluant followed by MeOH.

1, hexane/EtOAc(8:2); 2, hexane/EtOAc(6:4); 3, hexane/EtOAc (4:6); 4, hexane/EtOAc (2:8); 5, EtOAc 100%; 6, 100% MeOH.

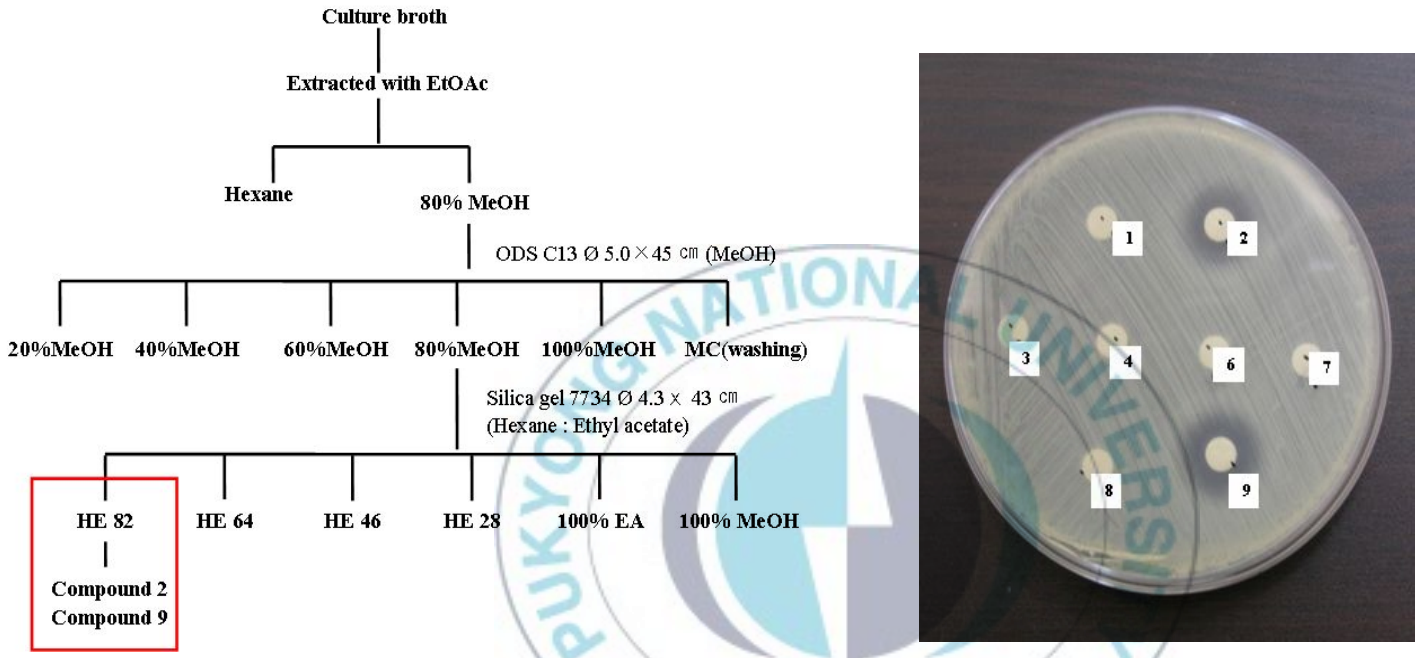


Fig. 21. The anti-MRSA activity of HPLC eluates from hexane / EtOAc (8:2) fraction. The compound was purified by reversed-phase HPLC (YMC ODS-A column, 10 × 250 mm; 60% MeOH in H_2O; flow rate, 1.5 ml/min; RI detector). 1, compound 1; 2, compound 2; 3, compound 3; 4, compound 4; 6, compound 6; 7, compound 7; 8, compound 8; 9, compound 9.

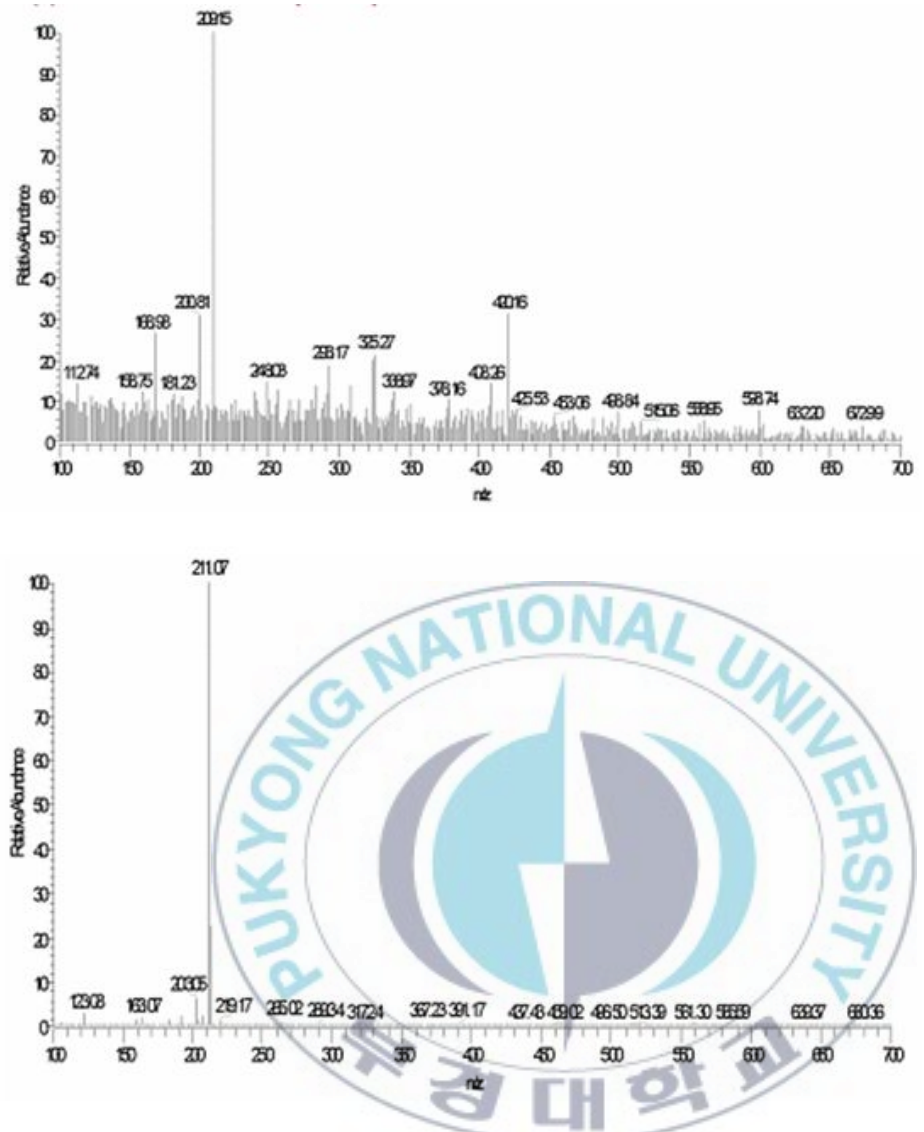


Fig. 22. LC-MS spectrum of the anti-MRSA compound (**1**). LC-mass spectrum of anti-MRSA compound indicated an intense peak at *m/z* 211.07. A, positive; B, negative.

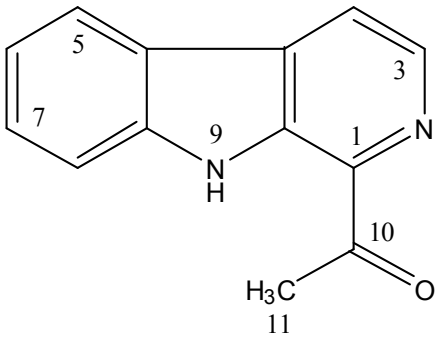


Fig. 23. The structure of anti-MRSA compound, 1-acetyl-β-corboline (**1**), isolated from *Pseudomonas* sp. UJ-6.

presence of β-carboline moiety (Fig. 22). The molecular formula of **1** was established as $C_{13}H_{10}N_2O$ by LC-MS and ^{13}C-NMR spectroscopic analyses (Table 10). The ^{1}H-NMR spectrum (Fig. 24) of **1** suggested that two ortho-coupled doublets at δ 8.45 and δ 8.30 (^{3}J = 4.8 Hz) indicated the presence of a heteroaromatic ring. Also, four multiplet protons at δ 8.21 (5-H), 7.58 (7-H), 7.70 (8-H), 7.30 (6-H) indicated 1,2-disubstituted aromatic ring and δ 2.80 peak indicated the presence of aromatic bound methyl group singlet. The ^{13}C NMR spectrum (Fig. 25) suggested that one acetyl carbonyl carbon (δ 203.3), five sp^2 quaternary carbons (δ 143.6, 138.7, 136.3, 133.4, 121.7), six aromatic methine carbons (δ 137.4, 130.4, 122.8, 121.7, 120.3, 113.6), and one methyl group sp^3 carbon (δ 25.8). The COSY (Fig. 26), gHSQC (Fig. 278) and gHMBC (Fig. 28) analyses of **1** led to reveal the structure of a 1-acetyl-β-carboline (Fig. 29). The identity of 1-acetyl-β-carboline was confirmed by the comparison with literature data of the chemical shifts. As is shown in Table 11, 1-acetyl-beta-carboline shows an antibacterial activity against MRSA strains tested with MICs ranging from 32 to 64 μg/ml.

β-carboline alkaloids, which are widespread in many plant families, are biosynthesized by microorganisms and they also occur in mammals (Jiménez *et al*., 2008). The activities of β-carboline alkaloids have

Table 10. ^{1}H- and ^{13}C-NMR spectroscopic assignment for 1-acetyl-β-carboline (**1**) in Methanol-d4

	Compound 2			
No.	δ_H	mult (*J* in Hz)	δ_C	HMBC
1			137.4	
2				
3	8.45	d (4.8)	138.7	C-1, -4, -4a
4	8.30	d (4.8)	120.3	C-3, -1a, -5a
4a			133.4	
5a			121.7	
5	8.21	dd (7.8)	122.8	C-7, -8a
6	7.30	td (7.8)	121.7	C-5a, -8
7	7.58	td (7.8)	130.4	C-5, -8a
8	7.70	dd (7.8)	113.6	C-5a, 6
8a			143.6	
1a			136.3	
9	10.30	s br.		
10			203.3	
11	2.81	s	26.2	C-1, -10

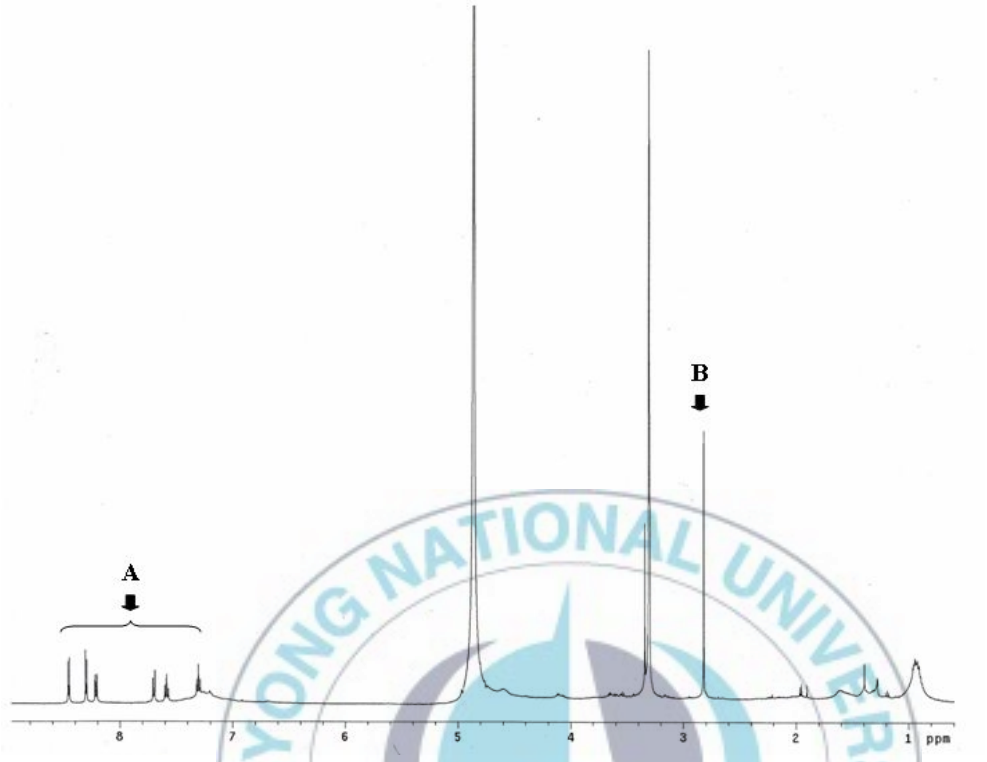


Fig. 24. ^{1}H-NMR spectrum of anti-MRSA compound (**1**) in CD_3OD-d_4 at 300 K. The arrow A and B indicate olefinic protons and aromatic bound methyl group singlet in the compound, respectively.

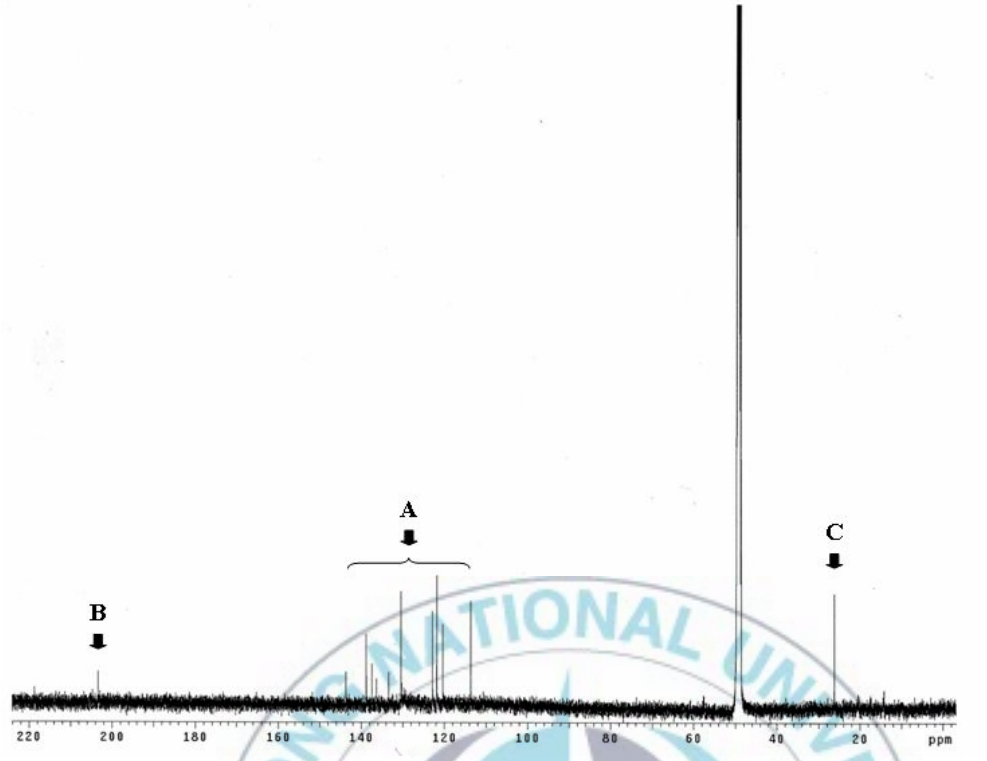


Fig. 25. ^{13}C-NMR spectrum of anti-MRSA compound (**1**) in CD_3OD-d_4 at 300 K. The arrow A, B, and C indicate aromatic carbons, one acetyl carbonyl carbon, and one methyl group sp^3 carbon in the compound, respectively.

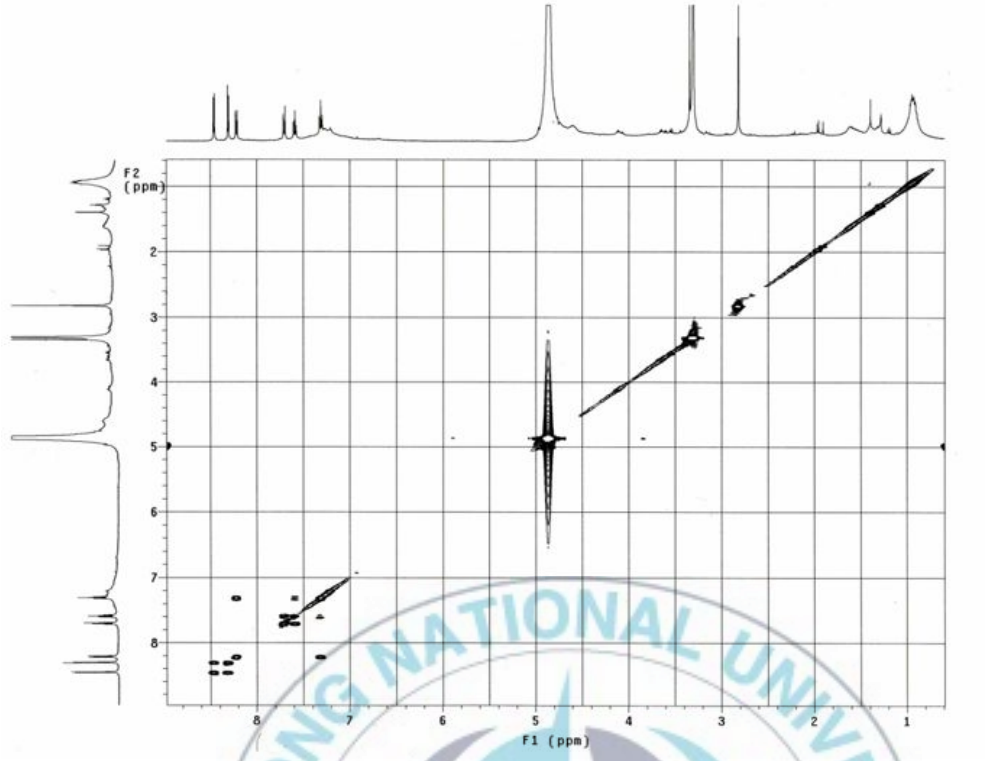


Fig. 26. ^{1}H-^{1}H COSY spectrum of anti-MRSA compound (**1**) in CD_3OD-d_4 at 300 K. The proton spectrum is presented along both the x-axis and the y-axis. Signals rise above the xy plane and are shown as a contour plot. Signals appear where one set of protons couples with another set of protons. This spectrum indicates which protons are coupled in a molecule.

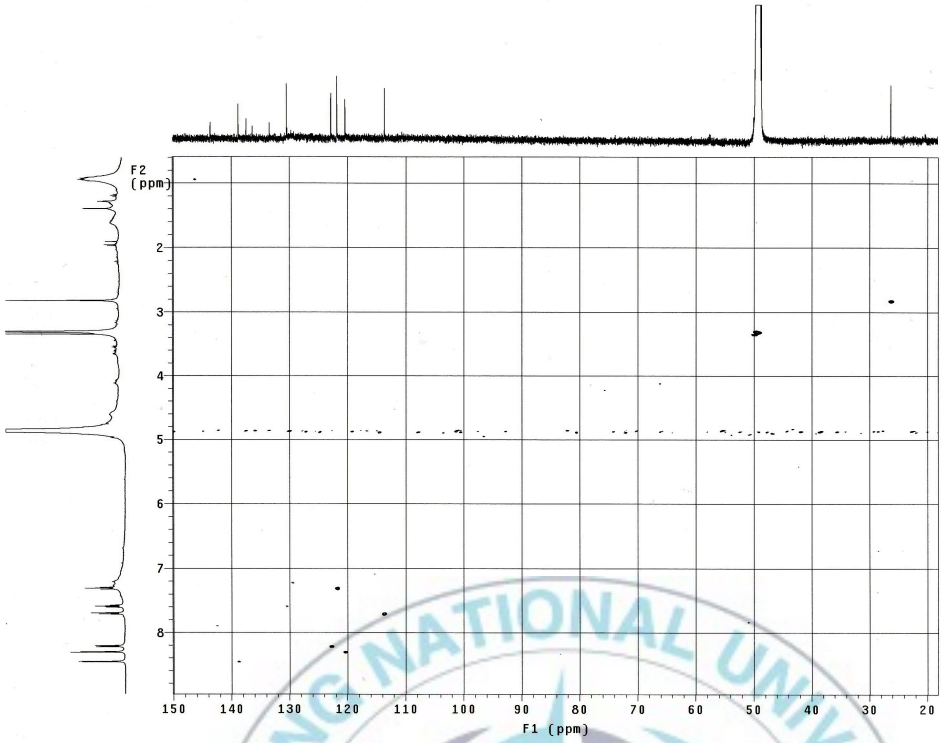


Fig. 27. ^{1}H-^{13}C gradient HSQC spectrum of anti-MRSA compound (**1**) in CD_3OD-d_4 at 300 K. This is a 2D experiment used to correlate, or connect, ^{1}H- and ^{13}C-peaks for directly bonded C-H pairs. The coordinates of each peak seen in the contour plot are the ^{1}H and ^{13}C chemical shifts.

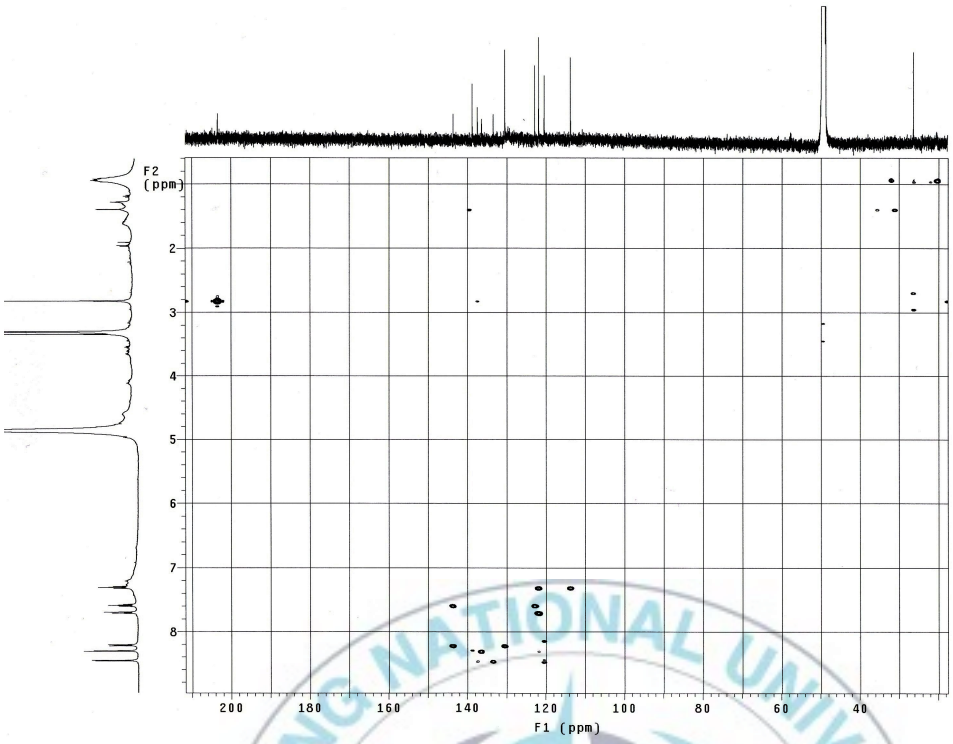
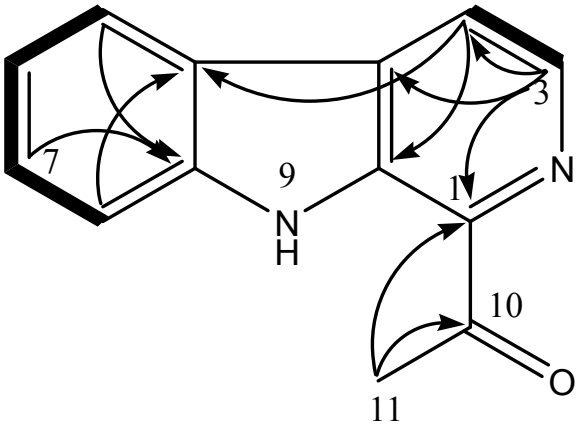


Fig. 28. Gradient HMBC spectrum of anti-MRSA compound (**1**) in CD_3OD-d_4 at 300 K. This is a 2D experiment used to correlate, or connect, ^{1}H and ^{13}C-peaks for atoms separated by multiple bonds (usually 2 or 3).The coordinates of each peak seen in the contour plot are the ^{1}H and ^{13}C chemical shifts.



: COSY correlaton

: HMBC correlaton

Fig. 29. Key HMBC and COSY correlations of 1-acetyl-β-carboline (**1**).

Table 11. MICs of 1-acetyl -beta-carboline against MSSA and MRSA

Strain	Source or reference	[b)]*mecA*	MIC (μg /ml)
			1-acetyl-beta-carboline
MSSA (KCTC 1927)	Standard strain	-	16
MRSA (KCCM 40510)	Standard strain	+	32
MRSA (KCCM 40511)	Standard strain	+	64
MRSA D-3	Clinical isolate[a)]	+	64
MRSA D-4	Clinical isolate	+	64
MRSA D-6	Clinical isolate	+	64
MRSA D-8	Clinical isolate	+	32
MRSA D-11	Clinical isolate	+	32
MRSA D-12	Clinical isolate	+	64
MRSA D-13	Clinical isolate	+	64
MRSA D-14	Clinical isolate	+	32
MRSA D-17	Clinical isolate	+	64
MRSA D-18	Clinical isolate	+	64

[a)] MRSA strains were isolated in Dong-A University Medical Hospital

[b)] +, *mec*A positive; -, *mec*A negative

been reported: inhibition of cytochrome P450 (Tweedie *et al*., 1988); inhibition of monoamine oxidase (Kim *et al*., 1997); binding to several serotonin, benzodiazepines and dopamine receptors (Glennon *et al*., 2000); and inhibition of DNA topoisomerase activities (Funayama *et al*., 1996). They have also anticancer (Jahaniani *et al*., 2005), antiviral, antibacterial, antifungal and algicidal (Volk and Furkert, 2006; Volk and Mundt, 2007) activity. 1-acetyl-β-carboline was first isolated from *Streptomyces kasugaensis* (Proksa *et al*., 1990) but there is little known about activities of 1-acetyl-β-carboline.

Chapter 4. Summary and Conclusion

MRSA is the most problematic gram-positive bacterium in public health because it has become resistant to almost all the currently available antibiotics except teicoplanin and vancomycin. With the increasing use of vancomycin application, its susceptibility to vancomycin has recently decreased and VISA and VRSA have been in number of countries. Thus, the development of new drugs or alternative therapies is clearly a matter of urgency.

Marine natural products provide a rich source of chemical diversity that can be used to design and develop new, potentially useful therapeutic agents. Certain marine products have been reported to exhibit antimicrobial effects against several pathogens. In an effort to discover an alternative antibiotic against MRSA, marine organisms are attracting attention because a few and novel antibiotics from marine organisms have been reported. The objectives of this study are to isolate and identify of anti-MRSA substances from marine organisms and to investigate the characteristics of the antibiotic active against MRSA.

In this study, it was identified an anti-MRSA substance, dieckol, from *E. stolonifera* and assessed its synergistic effects against MRSA when administered in combination with β-lactams. The dieckol purely isolated from *E. stolonifera* evidenced the antibacterial activity against

MSSA and MRSA with MIC values ranging from 32 to 64 ㎍/ml. Furthermore, dieckol clearly reversed the high- level ampicillin and penicillin resistance of MRSA. The MICs of ampicillin and penicillin combined with dieckol against the MRSA strains were dramatically reduced. Additionally, the FIC indices of ampicillin and penicillin against MRSA strains ranged between 0.066 and 0.266 when administered in combination with dieckol (8 or 16 ㎍/ml), thereby indicating the existence of a synergistic effect of dieckol-ampicillin or penicillin combinations against MRSA.

A marine bacterium, *Pseudomonas* sp. UJ-6, which exhibited an antibacterial activity against MRSA, was isolated. The culture broth and its ethyl acetate extract exhibited a bactericidal activity against MRSA, interfering with cell wall synthesis as does vancomycin. The extract also exhibited an antibacterial activity against Gram-negative bacteria, even though vancomycin was not effective against Gram-negative bacteria. The extract kept over 95% of its activity after heat treatment for 15 min at 121℃, indicating that a thermal stable antibiotic is produced by *Pseudomonas* sp. UJ-6, even though most known antibiotics are unstable to thermal stress. The active compound isolated from *Pseudomonas* sp. UJ-6 is identified to be 1-acetyl-β-carboline was confirmed by the comparison with literature data of the

chemical shifts. 1-acetyl-beta-carboline showed an antibacterial activity against MRSA strains tested with MICs ranging from 32 to 64 μg/ml.

The results of the present study are expected to contribute to the development of an alternative phytotherapeutic agent against MRSA, and in applications of the treatment of MRSA infections. Further study is needed to identify the rest of active compound and to investigate the characteristics of the anti-MRSA activity.

References

Acebal, C., L.M. Cañedo, J.L.F. Puentes, J.P. Baz, F. Romero, F. De La Calle, M.D.G. Grávalos, and P. Rodrigues. 1999. Agrochelin, a new cytotoxic antibiotic from a marine agrobacterium. Taxonomy, fermentation, isolation, physicochemical properties and biological activity. *J. Antibiot.* 52, 983–987.

Acebal, C., R. Alcazar, L.M. Cañedo, F. De La Calle, P. Rodriguez, F. Romero, and J.L.F. Puentes. 1998. Two marine agrobacterium producers of sesbanimide antibiotics. *J. Antibiot.* 51, 64–67.

Apfel C.M., H. Locher, S. Evers, B. Takacs, C. Hubschwerlen, W. Pirson, M.G.P. Page, and W. Keck. 2001. Peptide deformylase as an antibacterial drug target: target validation and resistance development. *Antimicrob. Agents Chemother.* 45, 1058-1064.

Arcus V.L., J.S. Lott, J.M. Johnston, and E.N. Baker. 2006. The potential impact of structural genomics on tuberculosis drug discovery. *Drug. Discov. Today* 11, 28-34.

Barna, J.C.J. and D.H. Williams. 1984.The structure and mode of action of glycopeptide antibiotics of the vancomycin group. *Annu. Rev. Microbiol.* 38, 339-357.

Bennamara, A., A. Abourriche, M. Berrada, M. Charrouf, N. Chaib, and M. Boudouma. 1999. Methoxybifurcarenone: an antifungal and antibacterial meroditerpenoid from the brown alga *Cystoseira tamariscifolia. Phytochemistry* 52, 37-40.

Biabani, M.A.F., H. Laatsch, E. Helmke, and H. Weyland. 1997. δ-Indomycinone: a new member of pluramycin class of antibiotics isolated from marine *Streptomyces* sp. *J. Antibiot.* 50, 874–877.

Black M.T. and J. Hodgson. 2005. Novel target sites in bacteria for

overcoming antibiotic resistance. *Adv. Drug Deliv. Rev.* 57, 1528-1538.

Blot, S.I., K.H. Vandewoude, E.A. Hoste, and F.A. Colardyn. 2002. Outcome and attributable mortality in critically Ill patients with bacteremia involving methicillin-susceptible and methicillin-resistant *Staphylococcus aureus*. *Arch. Intern. Med.* 162, 2229-2235.

Borysowski J., B. Weber-Dabrowska, and A. Gorski. 2006. Bacteriophage endolysins as a novel class of antibacterial agents. *Exp. Biol. Med. (Maywood)* 231, 366-377.

Caccamese, S., R. Azzolina, G. Furnari, M. Cormaci, and S. Grasso. 1981. Antimicrobial and antiviral activities of some marine algae from eastern Sicily. *Botanica Marina* 14, 365-367.

Carter, A.P., W.M. Clemons, D.E. Brodersen, R.J. Morgan-Warren, B.T. Wimberl, and V. Ramakrishnan. 2000. Functional insights from the structure of the 30S ribosomal subunit and its interactions with antibiotics. *Nature* 407, 340-348.

Centers for Disease Control and Prevention. 2007. MRSA: Methicillin-resistant *Staphylococcus aureus* in healthcare settings.

Chambers, H.F. 1995. In vitro and in vivo antistaphylococcal activities of L-695,256, a carbapenem with high affinity for the penicillin-binding protein PBP 2a, *Antimicrob. Agents Chemother.* 39, 462−466.

Chambers, H.F. 2001. The changing epidemiology of *Staphylococcus aureus*?. *Emerg. Infect. Dis.* 7, 178-182.

Chandrasekar, P.H., D.P. Levine, S. Price, and M.J. Rybak. 1988. Comparative efficacies of imipenem-cilastatin and vancomycin in

experimental aortic valve endocarditis due to methicillin resistant *Staphylococcus aureus*, *J. Antimicrob. Chemother.* 21, 461−469.

Cho, A., T.W. Glinka, M. Ludwikow, A.T. Fan, M. Wang, and S.J. Hecker. 2001. New anti-MRSA cephalosporins with a basic aminopyridine at the C-7 position, *Bioorg. Med. Chem. Lett.* 11, 137-140.

Clark, J., J.C. Fung-Tomc, B. Minassian, Y.H. Tsai, H. Yang, E. Huczko, B. Kolek, S. Chaniewski, C. Ferraro, R. Drain, E. Gradelski, and D.P. Bonner. 2002. In vitro and in vivo activities of a novel cephalosporin, BMS-247243, against organisms other than staphylococci. *Antimicrob. Agents Chemother.* 46, 1108-1111.

Clements J.M., A. Ayscough, K. Keavey, and S.P. East. 2002. Peptide deformylase inhibitors, potential for a new class of broad spectrum antibacterials. *Curr. Med. Chem*. 1, 239-249.

Coates A.RM. and Y. Hu. 2007. Novel approaches to developing new antibiotics for bacterial infections. *Br. J. Pharmacol.* 152, 1147-1154.

Cosgrove, S.E., Y. Qi, K.S. Kaye, S. Harbarth, A.W. Karchmer, and Y. Carmeli. 2005. The impact of Methicillin Resistance in *Staphylococcus aureus* Bacteremia on Patient Outcomes: Mortality, Length of Stay, and Hospital Charges. *Infect. Control Hosp. Epidemiol.* 26, 166-174.

Dabrowska K, K. Switala-Jelen, A. Opolski, B. Weber-Dabrowska, and A. Gorski. 2005. Bacteriophage penetration in vertebrates. *J. Appl. Microbiol.* 98, 7-13.

Dunbar, J., L.O. Ticknor, and C.R. Kuske. 2000. Assessment of

microbial diversity in four Southwestern United States soils by 16S rRNA gene terminal restriction fragment analysis. *Appl. Environ. Microbiol.* 66, 2943-2950.

Entenza, J.M., P. Hohl, I. Heinze-Krauss, M.P. Glauser, and P. Moreillon. 2002. BAL9141, a novel extended-spectrum cephalosporin active against methicillin-resistant *Staphylococcus aureus* in treatment of experimental endocarditis. *Antimicrob. Agents Chemother.* 46, 171-177.

Eom, S. H., M.S. Kang, and Y.M. Kim. 2008. Antibacterial ativity of the Phaeophyta *Ecklonia stolonifera* on methicillin-resistant *Staphylococcus aureus*. *J. Fish. Sci. Technol*. 11, 1-6.

Field D., J. Hughes, and E.R. Moxon. 2004. Using the genome to understand pathogenicity. *Methods Mol. Biol.* 266, 261-287.

Finland, M. 1979. Emergence of antibiotic resistance in hospital 1935-1975. *Rev. Infect. Dis.* 1, 4-21.

Fischetti V.A., D. Nelson, and R. Schuch. 2006. Reinventing phage therapy: are the parts greater than the sum? *Nat. Biotechnol.* 24, 1508-1511.

Forbes, B.A., K. Bombicino, K. Plata, A. Cuirolo, D. Webber, C.L. Bender, and A.E. Rosato. 2008. Unusual form of oxacillin resistance in methicillin-resistant *Staphylococcus aureus* clinical strains. *Diagn. Microbiol. Infect. Dis.* 61, 387-395.

Foster, T.J. 2004. The *Staphylococcus aureus* "superbug". *J. Clin. Invest.* 114, 1693–1696.

Fournier P.E., D. Vallenet, V. Barbe, S. Audic, H. Ogata, L. Poirel, H. Richet, C. Robert, S. Mangenot, C. Abergel, P. Nordmann, J.

Weissenbach, D. Raoult, and J.M. Claverie. 2006. Comparative genomics of multidrug resistance in *Acinetobacter baumannii*. *PLoS Genet.* 2, e7.

Funayama Y., K. Nishi, K. Wakabayashi, M. Nagao, K. Simio, T. Ohira, S. Hasegawa, and N. Saijo. 1996. Effects of β- and ɣ -carboline derivates on DNA topoisomerase activities. *Mutat. Res.* 349, 183-391.

Freiberg C and H. Brotz-Oesterhelt. 2005. Functional genomics in antibacterial drug discovery. *Drug Discov. Today* 10, 927-935.

Glennon R.A., M. Dukat, B. Brella, S.S. Hong, L. Constantino, M. Teitler, C. Smith, C. Egan, K. Davis, M.V. Mattson. 2000. Binding of β-carbolines and relating agents at serotonin (5-HT_2 and 5-HT_{1A}), dopamine (D_2) and benzodiazepines receptors. *Drug. Alcohol. Depend.* 60, 121-132.

Glinka, T.W. 2002. Novel cephalosporins for the treatment of MRSA infections, *Curr. Opin. Investig. Drugs* 3, 206-217.

Glinka, T., K. Huie, A. Cho, M. Ludwikow, J. Blais, D. Griffith, S. Hecker, and M. Dudley. 2003. Relationships between structure, antibacterial activity, serum stability, pharmacokinetics and efficacy in 3-(heteroarylthio) cephems. discovery of RWJ-333441 (MC-04, 546), *Bioorg. Med. Chem.* 11, 591-600.

Gerard, J.M., P. Haden, M.T. Kelly, and R.J. Andersen. 1999. Loloatins A-D, cyclic decapeptide antibiotics produced in culture by a tropical marine bacterium. *J. Nat. Prod.* 62, 80–85.

Guignard, B., J.M. Entenza, P. Moreillon. 2005. Beta-lactams against methicillin-resistant *Staphylococcus aureus*. *Curr. Opin. Pharmacol.*

5, 479-489.

Han, E.S., J.W. Kim,, M.O. Eom, I.H. Kang, H.J. Kang, J.S. Choi, K.W. Ha, and H.Y. Oh. 2000. Inhibitory effects of *Ecklonia stolonifera*on gene mutation on mouse lymphoma tk+/- locus in L5178Y-3.7.2C cell and bone marrow micronuclei formation in ddY mice. *Environ. Mutagen. Carcinogen.* 20, 104-111.

Hancock R.E.W. 2007. The end of an era? *Nat. Rev. Drug. Discov.* 6, 28.

Hanaki, H., H. Labischinski, T. Inaba, N. Kondo, H. Murakami, and K. Hiramatsu. 1998. Increase in glutamine-non-amidated muropeptides in the peptidoglycan of vancomycin-resistant *Staphylococcus aureus* strain Mu50. *J. Antimicrob. Chemother.* 42, 315-320.

Hara, I. and T. Sakurai. 1998. Isolation and characteriza-tion of vanadium bromoperoxidase from a marine macroalga, *Ecklonia stolonifera*. *J. Inorg. Biochem.* 72, 23-28.

Hardy, K.J., P.M. Hawkey, F. Gao, B.A. Oppenheim. 2004. Methicillin resistant *Staphylococcus aureus* in the critically ill. *Br. J. Anaesth.* 92, 121-130.

Hendrix R.W. 2002. Bacteriophages: evolution of the majority. *Theor. Popul. Biol.* 61, 471-480.

Hiramatsu K., H. Hanaki, T. Ino, K. Yabuta, T. Oguri, and F.C. Tenover. 1997. Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility. *J. Antimicrob. Chemother.* 40, 135−136.

Horikawa, M., T. Noro, and Y. Kamei. 1999. *In vitro*

antimethicillin-resistant *Staphylococcus aureus* activity found in extracts of marine algae indigenous to the coastline of Japan. *J. Antibiot.* 52, 186-189.

Hososaka, Y., H. Hanaki, H. Endo, Y. Suzuki, Z. Nagasawa, Y. Otsuka, T. Nakae, and K. Sunakawa. 2007. Characterization of oxacillin-susceptible *mec*A positive *Staphylococcus aureus*: a new type of MRSA. *J. Infect. Chemother.* 13, 79-86.

Imamura, N., M. Nishijima, T. Takadera, K. Adachi, M. Sakai, and H. Sano. 1997. New anticancer antibiotics, pelagiomicins produced by a new marine bacterium *Pelagiobacter variabilis*. *J. Antibiot.* 50, 8–12.

Isnansetyo, A., M. Horikawa, Y. Kamei. 2001. In vitro anti-methicillin-resistant *Staphylococcus aureus* activity of 2,4-diacetylphloroglucinol produced by *Pseudomonas* sp. AMSN isolated from a marine alga. *J. Antimicrob. Chemother*. 2001 47, 724-725.

Isnansetyo, A. and Y. Kamei 2003. MC21-A, a bactericidal antibiotic produced by a new marine bacterim, *Pseudoalteromonas phenolica* sp.O-BC30^{T}, against methcillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 47, 480-488.

Ito, T., Y. Katayama, K. Asada, N. Mori, K. Tsutsumimoto, C. Tiensasitorn, and K. Hiramatsu. 2001. Structural comparison of three types of staphylococcal cassette chromosome *mec* integrated in the chromosome in methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents. Chemother.* 45, 1323-1336.

Jahaniani F., S.A. Ebrahimi, N. Rahbar-Roshandel, and M.

Mahmoudian. 2005. Xanthomicrol is the main cytotoxic component of *Dracocephalum kotschyii* and a potential anti-cancer agent. *Phytochemistry*. 66, 1581-1592.

Jeong, S.I., W.S. Han, Y.H. Yun, and K.J. Kim. 2006. Continentalic aid from *Aralia continentalis* show activity against mthicillin-resistant *Staphylococcus aureus*. *Phytother. Res.* 20, 511-514.

Jevons, M.P. 1961. Celbenin-resistant staphylococci. *Br. Med. J.* 1, 124-125.

Jiménez J, L. Riverón-Negrete, F. Abdullaev, J. Espinosa-Aguirre, and R. Rodríguez-Arnaiz. 2008. Cytotoxicity of the beta-carboline alkaloids harmine and harmaline in human cell assays in vitro. *Exp. Toxicol. Pathol*. 60, 381-389.

Joe, M.J., S.N. Kim, H.Y. Choi, W.S. Shin, G.M. Park, D.W. Kang, and Y.K. Kim. 2006. The inhibitory effects of eckol and dieckol from *Ecklonia stolonifera* on the expression of matrix metalloproteinase-1 in human dermal fibroblasts. *Biol. Pharm. Bull.* 29, 1735-1739.

Kang, H.S., H.R. Kim, D.S. Byun, B.W. Son, T.J. Nam, and J.S. Choi. 2004. Tyrosinase inhibitors isolated from the edible brown alga *Ecklonia stolonifera*. *Arch. Pharm. Res.* 27, 1226-1232.

Kang, H.S., H.Y. Chung, J.H. Jung, B.W. Son, and J.S. Choi, 2003. A new phlorotannin from the brown alga *Ecklonia stolonifera*. *Chem. Pharm. Bull.* 51, 1012-1014.

Kim H., S.O. Sablin, and R.R. Ramsay. 1997. Inhibition of monoamine oxidase A by β-carboline de rivates. *Arch. Biochem.*

Biophys. 337, 137-142.

Kim, Y.C., R.B. An, N.Y. Yoon, T.J. Nam, and J.S. Choi. 2005. Hepatoprotective constituents of the edible brown alga *Ecklonia stolonifera* on tacrine-induced cytotoxicity in Hep G2 cells. *Arch. Pharm. Res*. 28, 1376-1380.

Klein, E, D.L. Smith, and R. Laxminarayan. 2007. Hospitalizations and Deaths Caused by Methicillin-Resistant *Staphylococcus aureus*, United States, 1999–2005. *Emerg. Infect. Dis.* 13, 1840-1846.

Klevens, R.M., M.A. Morrison, J. Nadle, S. Petit, K. Gershman, S. Ray, L.H. Harrison, R. Lynfield, G. Dumyati, J.M. Townes, A.S. Craig, E.R. Zell, G.E. Fosheim, L.K. McDougal, R.B. Carey, S.K. Fridkin.; for the Active Bacterial Core surveillance (ABCs) MRSA Investigators. 2007. Invasive Methicillin-Resistant *Staphylococcus aureus* Infections in the United States. *JAMA*. 298, 1763-1771.

Kluytmans, J., A. van Belkum, and H. Verbrugh. 1997. Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. *Clin. Microbiol. Rev.* 10, 505-520.

Kurata, K., K. Taniguchi, K. Shiraishi, and M.M. Suzuki. 1993. Ecklonialactones-C-F from the brown alga *Ecklonia stolonifera*. *Phytochemistry* 33, 155-159.

Larsen T.O., J. Smedsgaard, K.F. Nielsen, M.E. Hansen, and J.C. Frisvad. 2005. Phenotypic taxonomy and metabolite profiling in microbial drug discovery. *Nat Prod Rep.* 22, 672-695.

Lee, J.H., J.C. Park, and J.S. Choi. 1996. The antioxidant activity of *Ecklonia stolonifera*. *Arch. Pharm. Rev.* 19, 223-227.

Levy, S.B. 1998. Multidrug resistance-a sign of the times. *N. Engl. J.*

Med. 338, 1367-1368.

Levy, S.B. 2005. Antibiotic resistance-the problem intensifies. *Adv. Drug Deliv. Rev.* 57, 1446-1450.

Liu, C., C.J. Graber, M. Karr, B.A. Diep, L. Basuino, B.S. Schwartz, M.C. Enright, S.J. O'Hanlon, J.C. Thomas, F. Perdreau-Remington, S. Gordon, H. Gunthorpe, R. Jacobs, P. Jensen, G. Leoung, J.S. Rumack, and H.F. Chambers. 2008. A population-based study of the incidence and molecular epidemiology of methicillin-resistant *Staphylococcus aureus* disease in San Francisco, 2004-2005. *Clin. Infect. Dis.* 46, 1637-1646.

Lloyd, A.W. 1998a. Monitor: molecules and profiles. *Drug Discov. Today* 3, 480.

Lloyd, A.W. 1998b. Monitor: molecules and profiles, *Drug Discov. Today* 5, 122.

Lowy, F.D. 1998. *Staphylococcus aureus* infections. *N. Engl. J. Med.*, 339, 520-532.

Margolis P.S., C.J. Hackbarth, D.C. Young, W. Wang, D. Chen, Z. Yuan Z, R., and J. Trias. 2000. Peptide deformylase in Staphylococcus aureus: resistance to inhibition is mediated by mutations in the formyltransferase gene. *Antimicrob. Agents Chemother*. 44, 1825-1831.

Matsuda T., T.A. Freeman, D.W. Hilbert, M. Duff, M. Fuortes, P.P. Stapleton, and J.M. Daly. 2005. Lysis-deficient bacteriophage therapy decreases endotoxin and inflammatory mediator release and improves survival in a murine peritonitis model. *Surgery* 137, 639-646.

Mills S.D. 2006. When will the genomics investment pay off for antibacterial discovery? *Biochem. Pharmacol.* 71, 1096-1102.

Monaghan R.L. and J.F. Barrett. 2006. Antibacterial drug discovery-then, now and the genomics future. *Biochem. Pharmacol.* 71, 901-909.

Murakami, K., W. Minamide, K. Wada, E. Nakamura, H. Teraoka, and S. Watanabe. 1991. Identification of methicillin resistant strains of *staphylococci* by polymerase chain reaction. *J. Clin. Microbiol.* 29: 2240-2244.

Nagayama, K., Y. Iwamura, T. Shibata, I. Hirayama, and T. Nakamura. 2002. Bactericidal activity of phlorotannins from the brown alga *Ecklonia kurome. J. Antimicrob. Chemother*. 50, 889-893.

Nascimento G.G.F., J. Locatelli, P.C. Freitas, and G.L. Silva. 2000. Antibacterial activity of plant extracts and phytochemicals on antibiotic-resistant bacteria. *Brasil. J. Microbiol.* 31, 246-256.

National Committee for Clinical Laboratory Standards. 2004. Method for dilution antimicrobial susceptibility testing for bacteria that grow aerobically. Approved standard M7-A6, 7th ed. National Committee for Clinical Laboratory Standards, Wayne, PA, USA.

Nicolas, M.H., M.D. Kitzis, and A. Karim. 1993. Stérilisation par 2 grammes d'Augmentin (R) des urines infectées à *Staphylococcus aureus* résistant à la méticilline. *Med. Mal. Infect.* 23, 82-94.

Norden, C.W., H. Wentzel, and E. Keleti. 1979. Comparison of techniques of measurement of in vitro antibiotic synergism. *J. Infect. Dis.* 140, 629-633.

Noskin, G.A., R.J. Rubin, J.J. Schentag, J. Kluytmans, E.C. Hedblom, M. Smulders, E. Lapetina, and E. Gemmen. 2005. The Burden of *Staphylococcus aureus* Infections on Hospitals in the United States: An Analysis of the 2000 and 2001 Nationwide Inpatient Sample Database. *Arch. Intern. Med.* 165, 1756-1761.

Ohtake, N., H. Imamura, H. Jona, H. Kiyonaga, A. Shimizu, M. Moriya, H. Sato, M. Nakano, R. Ushijima, and S. Nakagawa. 1998. Novel dithiocarbamate carbapenems with anti-MRSA activity. *Bioorg. Med. Chem.* 6, 1089-1101.

Park, J.Y., J.S. Jin, H.Y. Kang, E.H. Jeong, J.C. Lee, Y.C. Lee, S.Y. Seol, D.T. Cho, and J. Kim. 2007. A comparison of adult and pediatric methicillin-resistant *Staphylococcus aureus* isolates collected from patients at a university hospital in Korea. *J. Microbiol.* 45, 447-452.

Polissi A. and M.R. Soria. 2005. Functional genomics of bacterial pathogens: from post-genomics to therapeutic targets. *Mol. Microbiol.* 57, 307-312.

Proksa B., D. Uhrin, M. Sturdikova, and J. Fuska. 1990. 1-Acetyl-β -carboline, a new metabolite of *Streptomyces kasugaensis. Scta. Biotechnol.* 10, 337-340.

Pusecker, K., H. Laatsch, E. Helmke, and H. Weyland. 1997. Dihydrophencomycin methyl ester, a new phenazine derivative from a marine *Streptomycete*. *J. Antibiot.* 50, 479–483.

Sakharkar K.R., M.K. Sakharkar, and V.T. Chow. 2004. A novel genomics approach for the identification of drug targets in pathogens, with special reference to *Pseudomonas aeruginosa*. *In*

Silico. Biol. 4, 355-360.

Sakon, J., H.H. Liao, A.M. Kanikula, M.M. Benning, I. Rayment, and H.M. Holden. 1993. Molecular structure of kanamycin nucleotidyl transferase determined to 3.0-A resolution. *Biochemistry* 32, 11977-11984.

Schaberg, D.R., D.H. Culver, and R.P. Gaynes. 1991. Major trends in the microbial etiology of nosocomial infection. *Am. J. Med.* 91, 72S-75S.

Schmitt-Hoffmann, A., L. Nyman, B. Roos, M. Schleimer, J. Sauer, N. Nashed, T. Brown, A. Man, and E. Weidekamm. 2004. Multiple-dose pharmacokinetics and safety of a novel broad-spectrum cephalosporin (BAL5788) in healthy volunteers, *Antimicrob. Agents Chemother.* 48, 2576-2580.

Shioda, S., M. Shimizu, J. Sugiyama, Y. Morita, T. Mizushima, and T. Tsuchiya. 2004. Mechanisms of action of corilagin and tellimagrandin I that remarkably potentiate the activity of β-lactams against methicillin-resistant *Staphylococcus aureus*. *Microbiol. Immunol.* 48, 67-73.

Sulakvelidze A, Z. Alavidze, and J.G. Morris. 2001. Bacteriophage therapy. *Antimicrob. Agents Chemother*. 45, 649-659.

Sumita, Y., H. Nouda, K. Kanazawa, and M. Fukasawa. 1995. Antimicrobial activity of SM-17466, a novel carbapenem antibiotic with potent activity against methicillin-resistant *Staphylococcus aureus. Antimicrob. Agents. Chemother.* 39, 910–916.

Swenson, J.M., D. Lonsway, S. McAllister, A. Thompson, L. Jevitt, W. Zhu, and J.B. Patel. 2007. Detection of *mec*A-mediated

resistance using reference and commercial testing methods in a collection of *Staphylococcus aureus* expressing borderline oxacillin MICs. *Diagn. Microbiol. Infect. Dis.* 58, 33-39.

Taga, N. 1968 Some ecological aspects of marine bacteria in the Kuroshio Current. *Bull. Misaki Mar. Biol. Kyoto Univ*. 12, 65-76.

Tan Y.T., D.J. Tillett, and I.A McKay. 2000. Molecular strategies for overcoming antibiotic resistance in bacteria. *Mol. Med. Today*. 6, 309-314.

Taniguchi, K., K. Kurata, and M. Suzuki. 1991. Feeding-deterrent effect of phlorotannins from the brown alga *Ecklonia stolonifera* against the abalone *Haliotis discus hannai. Nippon Suisan Gakkaishi* 57, 2065-2071.

Tweedie D.J., R.A. Prough, and M.D. Burke. 1998. Effects of induction on the metabolism and cytochrome P-450 binding of harman and other β-carbolines. *Xenobiotica* 18, 785-796.

UK Office for National Statistics. 2007. MRSA deaths continue to rise in 2005.

Volk, R.B. and F.H. Furkert. 2006. Antialgal, antibacterial and antifungal activity of two metabolites produced and excreted by cyanobacteria during growth. *Microbiol. Res.* 161, 180–186.

Volk, R.B. and S. Mundt. 2007. Cytotoxic and non-cytotoxic exometabolites of the cyanobacterium *Nostoc insulare*. *J. Appl. Phycol.* 19, 55–62.

Watanabe, A., Y. Tokue, H. Takahashi, T. Kikuchi, T. Kobayashi, K. Gomi, S. Fujimura, S. Yasui, Y. Murayama, and T. Nukiwa. 2001. Comparative in-vitro activity of carbapenem antibiotics against

respiratory pathogens isolated between 1999 and 2000, *J. Infect. Chemother.* 7, 267-271.

Witte, W. 1999. Antibiotic resistance in gram-positive bacteria: epidemiological aspects. *J. Antimicrob. Chemother.* 44, 1-9.

Witte, W, B. Pasemann, and C. Cuny. 2007. Detection of low-level oxacillin resistance in *mec*A-positive *Staphylococcus aureus*. *Clin. Microbiol. Infect.* 13, 408-412.

Wyllie, D., D. Crook, and T. Peto. 2006. Mortality after *Staphylococcus aureus* bacteraemia in two hospitals in Oxfordshire, 1997−2003: cohort study. *BMJ* 333, 269-270.

Yuan Z and R.J. White. 2006. The evolution of peptide deformylase as a target: contribution of biochemistry, genetics and genomics. *Biochem. Pharmacol.* 71, 1042-1047.

Zhao, W.H., Z.Q. Hu, S. Okubo, Y. Hara, and T. Shimamura. 2001. Mechanism of synergy between epigallocatechin gallate and β-lactams against methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 45, 1737-1742.

Isolation and Characterization of Anti-MRSA (Methicillin-Resistant *Staphylococcus aureus*) Substances from Marine Organisms

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요 약

현재 병원 감염의 주 원인균이며 기존 항생제에 다제내성인 MRSA가 문제되고 있다. 이에 대한 MRSA의 치료제로 반코마이신이 사용되고 있으나 그 사용이 아주 제한적이며 최근에 반코마이신에 대한 내성 균주가 출현하여 새로운 항생제 개발이 요구되고 있다. 해양 생물의 경우에는 토양과 달리 그 서식처가 특수 서식환경으로 지금까지 알려지지 않았던 생리기능성 물질들을 가지고 있으며 현재 많은 보고가 되고 있다. 이러한 해양 유래 천연물들은 새로운 항생제 개발 등에 대한 많은 가능성을 제시해주고 있으며 해양 생물로부터 MRSA에 대하여 뛰어난 항균력을 가진 물질을 분리하게 된다면 새로운 구조나 작용 메카니즘을 가진 새로운 항생물질일 가능성이 높다.

따라서 본 연구에서는 해양생물체로부터 MRSA에 대한 항균력을 가지는 물질을 분리하고 그 물질의 항균 특성 등에 대하여 조사하였다. 갈조류인 *Ecklonia stolonifera* 로부터 분리한 dieckol은 MRSA에 대해 항균효과를 가졌으며 MIC 32~64 μg/ml의 값을 나타내었다. *E. stolonifera* 로부터 분리한 dieckol과 β-lactam계 항생제인 ampicillin과 penicillin과의 병용사용 실험결과 이 두 항생제에 대해 높은 synergy 효과를 가지는 것을 알 수 있었다. MRSA에 대한 항균력을 나타내는 해양 세균을 탐색한 결과 *Pseudomonas* sp. UJ-6을 분리할 수 있었다. *Pseudomonas* sp. UJ-6 로부터 가추출한 ethyl acetate extract는 높은 열처리에도 불구하고 95%이상의 활성을 유지하였으며 MRSA의 세포벽을 파괴시키는 것을 확인할 수 있었다. 또한 *Pseudomonas* sp. UJ-6 로부터 MRSA에 대한 항균효과를 가지는 eudistomin 계열의 1-acetyl-beta-carboline을 분리하였으며 MRSA에 대한 MIC는 32~64 μg/ml의 범위로 나타났다. 이상의 결과로부터 선발된 물질들은 MRSA에 대한 치료제 개발에 있어서 유용하게 이용될 수 있을 것이며, 산업적 이용 가능성도 클 것으로 사료된다.

감사의 글

이 논문을 완성하기까지 많은 관심과 아낌없는 사랑으로 도움을 주셨던 모든 분들께 진심으로 감사드립니다. 제가 해양미생물 연구실에서 실험을 배운지 10년이 되었습니다. 힘들고 어려웠던 시간이었지만 돌아보면 제 자신에게 정말 소중하고 중요한 시간이었으며 더욱더 발전하며 노력하는 사람이 되도록 하겠습니다.

한없이 부족한 저를 끝없는 이해와 사랑으로 이끌어주시고 저의 미비한 실험이 이렇게 논문으로 완성되기까지 지도 편달을 해주신 이원재 교수님께 먼저 머리숙여 감사드립니다. 그리고 부족한 논문을 정성껏 다듬어 주시고 논문수정의 수고를 아끼지 않으신 이명숙 교수님, 송영환 교수님, 신라대 손재학 교수님, 식품공학과 김영목 교수님께 감사드리며 학문의 바른길을 제시해주신 김진상 교수님, 이훈구 교수님, 김영태 교수님 최태진 교수님, 김군도 교수님께도 깊은 감사를 드립니다.

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교수님, 정원교 교수님, 이상훈 선배님, 제재영 교수님, 엄성환 후배님, 엄태길 선생님께도 감사드립니다.

오랜 시간 같은 공간에서 성격 나쁜 선배 기분 맞추며 공부하느라 힘들었을 윤숙.. 졸업 축하하며 고생 많으셨습니다. 항상 친구처럼 곁에서 응원해주며 힘이 되어준 용배, 정환, 인영.. 정말 고맙고 곧 좋은 결실 얻으시길 빌겠습니다. 한 동안 실험실 생활과 대학원 생활을 같이 했던 이무형 박사님, 김경석 선배님, 윤홍묵 선배님, 안영욱 선배님, 종오, 동욱, 지현, 주연, 지연, 재경, 경규, 영주, 선미, 미영, 혜영, 근호, 경용, 다영, 혜진, 혜경, 상은, 성석, 철우, 태혁, 지원, 경란.. 같이 한 시간들 아름답게 간직하겠습니다. 허드렛일도 마다않고 도와준 은주, 현순, 영주, 고은, 이번에 기나긴 석사과정을 마치는 희정이에게도 고마움 전합니다. 돈독한 우정으로 곁에서 응원해준 미르회 친구 원기, 은정, 원, 성우, 홍민, 민수, 현종이와 유학 공부중인 재광이와 보경, 미비한 문서에도 짜증내지 않고 도와주던 희주, 힘든 여정 속에 같이 학위를 수여하시는 김성현 선생님, 그리고 일일이 이름을 나열하지 못했지만 믿음으로 지켜봐 주시고 격려해 주신 모든 분들께도 다시 한번 감사드립니다.

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