# Mechanistic analysis of novel therapeutically effective antimicrobial agents identified using silkworm bacterial infection model

（カイコ細菌感染モデルを用いて同定された治療効果を示す新規抗菌薬の機能解析）

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Degree of Doctor of Philosophy

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*Atmika Paudel*

*January 2013*

**ABSTRACT**

**Mechanistic analysis of novel therapeutically effective antimicrobial agents identified using silkworm bacterial infection model**

**Introduction**

Emergence of resistant bacterial strains shortly after the clinical use of antibiotics has made infections difficult to treat. In order to continuously overcome the infectious diseases, discovery of novel antimicrobial agents with novel mechanism of action is utmost important. Despite the need of the novel antimicrobial agents, less attention is paid by pharmaceutical industries on this field due to poor outcomes. Although many screening programs have attempted to identify antimicrobial agents, the discovery of therapeutically effective novel compounds is very difficult and has not been reported in recent years. This can be attributed to the conventional method of antibiotic discovery, *in-vitro* screening followed by *in-vivo* screening, where ethical issues make it difficult for the use of animal models at the early stage of drug development. In fact, the use of mammalian models to examine the pharmacodynamics requires larger and sophisticated space, skilled personnel; is costly and associated with ethical issues. To address these issues, I propose use of silkworm infection model for identification of novel therapeutically effective antimicrobial agents. Silkworm model not only reduces the time and cost of experiments, but also requires less space and there are no ethical issues surrounding its use. Here, I summarize the identification of two novel therapeutically effective antimicrobial agents: kaikosin E, and compound 363, which were obtained from culture broth of a lysobacter strain and a chemical library, respectively, by using silkworm bacterial infection model. Moreover, a detailed insight on the identification of mechanism of action of these antimicrobial agents is provided. Whenever a new compound is identified that is therapeutically effective and has a potential to be used for clinical purposes, the elucidation of mechanism becomes important. The elucidation of mechanism of action of such compounds not only helps in understanding the pharmacokinetics, pharmacodynamics and fate of drug molecule, but also helps in reducing treatment failure.

**Results**

**I. Kaikosin E**

***a. Characterization of kaikosin E:*** Kaikosin E, a novel therapeutically effective antibiotic, was isolated from culture supernatant of a lysobacter species by using silkworm infection model. Kaikosin E was effective against silkworm infected with *Staphylococcus aureus* with an effective dose fifty ($ED_{50}$) value of 0.3 μg/g•larva. It was also effective in mouse infection model with an $ED_{50}$ value of 0.6

mg/kg, more potent than vancomycin. The structure of kaikosin E was elucidated by MS/MS and NMR analyses as shown in **Figure 1**. Kaikosin E is a cyclic lipopeptide containing 12 amino acid residues and a short fatty acid chain with a molecular mass of 1617. It was effective against Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA) with minimum inhibitory concentration (MIC) value of 4 µg/ml and was also effective against Mycobacterium (MIC: 8 µg/ml). Kaikosin E inhibited the biosynthesis of the macromolecules DNA, RNA, protein, and peptidoglycan in exponentially growing *S. aureus*. It exerted bacteriolytic activity and showed bactericidal activity with killing of 99% of bacteria within one minute of exposure to it. It dissipated the membrane potential in *S. aureus* even at concentrations much lower than that of MIC value suggesting the potent membrane damaging effect **(Figure 2).** Mice did not die when injected with a dose of 400 mg/kg kaikosin E, i.e., the ratio of lethal dose fifty to effective dose fifty ($LD_{50}/ED_{50}$) was more than 600 indicating the wide range of therapeutic index and low toxicity. Based on its therapeutic activity and low toxicity, kaikosin E has a strong potential to be a candidate for clinical application.

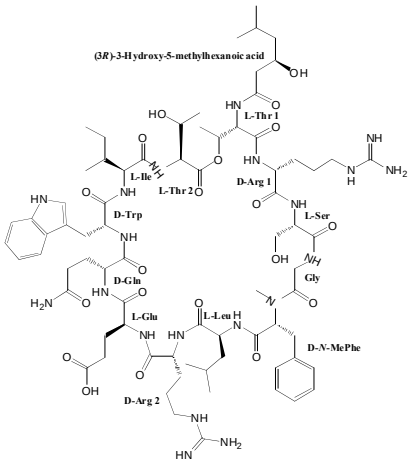



**Figure 1**

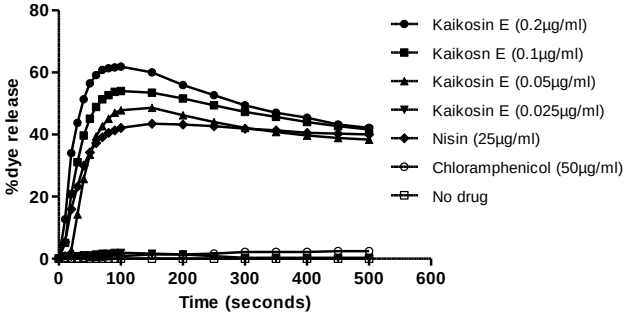



**Figure 2**

***b. Cellular target of kaikosin E in S. aureus***

To reveal the cellular target of kaikosin E, I isolated mutants of *S. aureus* resistant to it. *S. aureus* strain RN4220 was treated with a mutagen ethyl methanesulfonate and cultured on agar plates with different concentrations of kaikosin E. Resistant mutants that grow at 30°C but do not grow at 43°C, referred as temperature sensitive (TS) mutants, were further selected from the strains resistant to kaikosin E. I took advantage of the fact that TS phenotype correlates with mutations in essential genes. Usually, the mutations conferred are point mutations leading to change in an amino acid sequence of a protein, the stability of protein becomes dependent upon the temperature and the protein cannot function at higher temperatures but can still function at lower temperatures. This strategy helped to ignore other nonessential mutations that are less likely related to the target. I identified two TS strains resistant to kaikosin E. Insertion of DNA library and analysis of these two TS

strains revealed the presence of point mutations in *fni* (SA1236) and *menA* (SA0894), respectively. Both of the genes are essential genes required for menaquinone biosynthesis, a key component of respiration in *S. aureus* (**Figure 3)**. Since *fni* and *menA* genes were mutated independently in two strains, wild-type *fni* or *menA* were inserted in the respective strain, which complemented the TS phenotype in each strain suggesting that these two mutations were responsible for the TS phenotype of the mutants. Mutations in these two genes in kaikosin E resistant strains and involvement of these genes in the menaquinone biosynthetic pathway led to the speculation that menaquinone might be

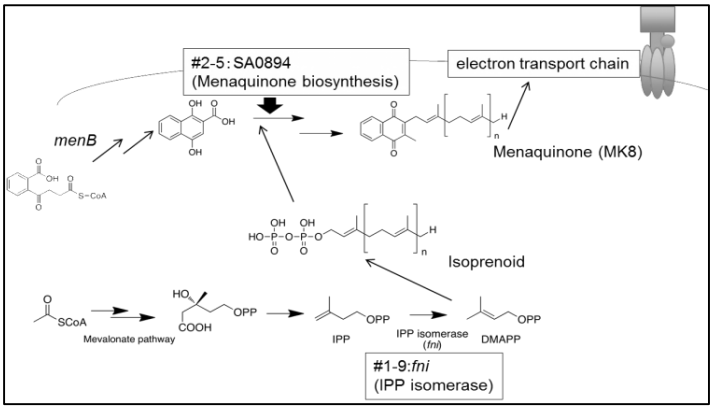



**Figure 3**

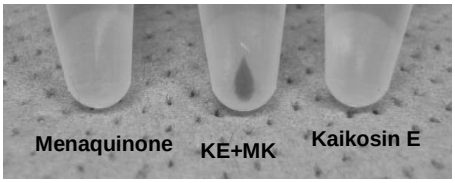



**Figure 4**

the target of kaikosin E. This was further evident from direct binding of kaikosin E to menaquinone (**Figure 4**). Furthermore, antimicrobial activity of kaikosin E attenuated upon addition of external menaquinone. The results unequivocally mentioned that menaquinone is the cellular target of kaikosin E. Since menaquinone does not exist in mammalian cells where its function is replaced by ubiquinone, the effect of addition of external ubiquinone on the antimicrobial activity of kaikosin E was tested. No change in the activity of kaikosin E upon addition of ubiquinone further provided the evidence for selectivity of kaikosin E towards microorganisms. This is the first report to reveal menaquinone as the target of any antimicrobial agents till date.

**II. Compound 363**

Another novel antimicrobial agent, compound 363 was discovered from chemical library of about 100,000 compounds screened on the basis of therapeutic effect in silkworm infection model. To date, compound 363 is not known to have antimicrobial activity. It showed antibacterial activity against methicillin-susceptible *S. aureus* (MSSA) and MRSA with MIC value of 6.3 μg/ml and was bacteriostatic. It showed therapeutic activity in silkworm infection model with $ED_{50}$ value of 39 μg/g•larva. It inhibited RNA

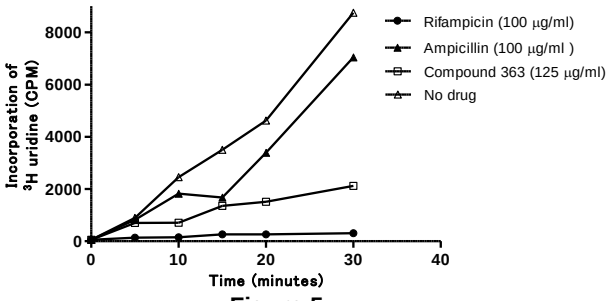



**Figure 5**

synthesis in *S. aureus* (**Figure 5**). TS mutants resistant to compound 363 were obtained and their whole genome was sequenced by a next generation sequencer to identify mutated gene responsible for the resistance. The *sigA* gene encoding RNA polymerase sigma factor was found to have a point mutation among two of the resistant mutants. *sigA* is an essential gene responsible for initiation of transcription of house-keeping genes. The mutation in the *sigA* gene explained the result of inhibition on RNA synthesis in *S. aureus* by this compound. Phage transduction was performed to check if the mutation in *sigA* was responsible for resistance to compound 363. From the two resistant strains, 78% and 87% of the transductants were susceptible to compound 363, which is close to the calculated expected value of 85% **(Table 1).**

**Table 1:** Susceptibility of transductants to compound 363

| Transductants from strain | Susceptible to compound 363 |
|---|---|
| # 74 | 78% |
| # 108 | 87% |

The whole genome sequence data suggested that no other gene close to *sigA* gene had mutations in both the strains. This, together with the phage transduction results, suggests that mutation in *sigA* gene is responsible for resistance conferred to compound 363.

**Discussion**

Based on the silkworm infection model, two novel therapeutically effective antimicrobial agents were identified. A novel strategy of TS screening was applied to find the target of kaikosin E as menaquinone. Lack of menaquinone in mammals explained its selective toxicity towards microorganisms and showed that kaikosin E has a great potential for clinical applications. This is the first report to reveal menaquinone as a target of an antibiotic. For compound 363, mutation in *sigA* gene was found to be responsible for resistance conferred to this compound suggesting that RNA polymerase sigma factor might be the target and involved in the mechanism of antibacterial action of compound 363. The mechanism found from this study can be further exploited to the screening, identification and design of novel therapeutically effective drug molecules ultimately providing novel insights for development of novel antimicrobial agents. These findings showed that silkworm infection model can be applied to identify novel therapeutically effective antimicrobial agents.

**Publications**

Paudel A, Hamamoto H, Kobayashi Y, Yokoshima S, Fukuyama T, and Sekimizu K. Identification of novel deoxyribofuranosyl indole antimicrobial agents. Journal of Antibiotics (Tokyo), 65:53-57 (2012).

Sekimizu N, Paudel A, and Hamamoto H. Animal welfare and use of silkworm as a model animal. Drug Discovery and Therapeutics, 6:226-229 (2012).

# INTRODUCTION

**Emergence of drug resistant pathogens: A global challenge**

The growing incidence of drug resistant pathogenic bacteria is a global threat. The rate of emergence of pathogenic bacteria resistant to currently available antimicrobial agents is higher than the rate of identification of novel antimicrobials. Due to this reason, resistance to currently available antimicrobials has become one of the most challenging problems and we are left with a limited number of therapeutic alternatives. If the same trend continues, many human infectious diseases will be untreatable in the near future. Strains intermediate to vancomycin, the so-called last resort for methicillin resistant *Staphylococcus aureus* (MRSA), already emerged as early as 1997 in Japan (Hiramatsu et al., 1997) and vancomycin resistant Enterococcus (VRE) emerged in 1987 (Leclercq et al., 1988) only a few years after its worldwide use (Moellering, 2006). Strains resistant to other newly discovered antibiotics effective against MRSA and VRE; linezolide and daptomycin, have already emerged within a couple of years of their clinical use (Tsiodras et al., 2001; Mangili et al., 2005; and Marty et al., 2006). Apart from this, the extended use of broad-spectrum antibiotics might also contribute to some level for development of resistance as they inhibit growth of a wide range of bacteria (Kollef MH, 2008). Another problem with the prolonged use of broad spectrum antibiotics is that the normal flora of human body will also be effected which can increase the chances for opportunistic bacteria to infect the body more easily, and in immunocompromised states, rare microorganisms can act as pathogens (Rosamond and Allsop, 2000). Therefore, the better clinical outcome is observed by changing the empirical use of broad-spectrum antibiotics on a rotation basis and switching from broad spectrum to narrow spectrum antibiotics as soon as the causative organism has been identified (Raymond et al., 2001; Kollef MH, 2008).

Hence, finding new antimicrobials, preferably narrow spectrum, should be a top priority in order to be able to treat notorious infectious diseases. Even though, the attempts to find new antimicrobial agents are centuries old, the challenge associated with it remains the same. Man has exploited nature as well as created thousands of synthetic chemicals in an attempt to alleviate infections.

**Approaches for identifying novel antimicrobial agents**

The empirical method of searching antimicrobial agents is screening for *in-vitro* antimicrobial activity against pathogens. If the source of screening is the library of crude extracts, the crude fraction is further processed to purification to obtain the active compound. Once an active compound has been obtained, it is tested for *in-vivo* therapeutic effects using mice or rats. However, not all *in-vitro* effective compounds show *in- vivo* effects. This is true basically because the *in-vitro* assays usually do not reflect true clinical situations as they differ from the *in-vivo* condition where there is a dynamic exposure of the infective agent to the antibiotic. The pharmacodynamics and pharmacokinetics parameters make many *in-vitro* effective compounds to be ineffective *in-vivo*. On the basis of this fact, a lot of time and effort is required to get a therapeutically effective compound by this method. Some scientists have tried to mimic the *in-vivo* situation with pharmacokinetic- pharmacodynamic approaches (Mueller et al., 2004; Tomita et al., 2007; Budha et al., 2009) which still does not reflect to the real *in-vivo* situation with many variables such as protein binding, tissue distribution, metabolism and other pharmacokinetic and pharmacodynamic parameters as well as toxicity. Therefore, selective, efficient and effective screening system is desired that can lead to identification of therapeutically effective drug seeds in less time.

Another problem with the empirical method is that this method requires many mammals to be sacrificed. Use of mammalian animals is associated with several problems: high cost, long breeding time, large space requirement and above all of these: the ethical issue (Baumans, 2004). Recently, interest towards use of invertebrate animal models is increasing due to problems to use large number of mammalian animals. Though tests in mammals are essential before the clinical trials of any candidate drug molecule, for the initial stage of tests, invertebrates are preferable. Invertebrates can be directly used for initial screening immediately after the *in-vitro* tests so that the experiments can be performed in an *in-vivo* setting at the initial stage. Several invertebrates have been applied in many scientific studies, some of them are: round worm *Caenorhabditis elegans* (Mahajan-Miklos, 1999), fruit fly *Drosophila melanogaster* (Lemaitre et al., 1996; Bernal and Kimbrell, 2000), grasshopper *Romalea microptera* (Johny et al., 2007), wax moth larva *Galleria mellonella* (Champion et al., 2009), silkworm larva *Bomboyx mori* (Kaito et al., 2002). Although some problems cannot be avoided with the use of invertebrates, many advantages outweigh their disadvantages. Some of the advantages are: they are cost-effective, can be easily handled, there are no ethical issues surrounding their use, and many biological processes and genes are conserved between mammals and invertebrates.

**Advantages of silkworm infection model**

Among the invertebrate animals, silkworms are preferred animal model because of many advantages. The body size of silkworms is large enough for easy handling and carrying out experiments that require organ isolation. Also, administration of accurate dose of sample is possible owing to its size. Due to the long and established breeding history and easy

availability and accessibility to silkworm eggs and artificial diet, it is possible to breed them all round the year. The established rearing method and well controlled breeding system is also an added point that gives silkworms of same genetic makeup and avoids individual variations which is crucial in obtaining reproducible and reliable results. Genome sequence of silkworm is already known (Mita et al., 2004) making easier for its genetic modification and producing transgenic silkworms. Similarity in host responses and virulence effects in silkworms and mammals makes it an acceptable model for infection related experiments. Use of silkworm is not bothered by ethical issue and this makes it possible to use them for initial stage of screening thus helping to find pharmacokinetically, pharmacodynamically acceptable and therapeutically effective drug seeds. This can help in discovery of therapeutically effective molecules that are otherwise ignored and/or missed by the *in-vitro* methods. Use of silkworms dramatically reduced the number of mammals for the experiments thus protecting the animal welfare (Sekimizu et al., 2012).

**GOALS OF THE STUDY**

1. Identification of therapeutically effective antimicrobial agents using silkworm infection model and elucidation of the mechanism of action
2. Identification of novel antimicrobial agents against drug resistant bacteria

# PART 1

## Identification of novel therapeutically effective antimicrobial agents using silkworm bacterial infection model

## Background

It has been found that the pathogenic microorganisms exerted their pathogenicity and killed silkworm larvae but not non-pathogenic microorganisms (Kaito et al, 2002). Lethal dose fifty ($LD_{50}$) values of bacterial toxins and cytotoxic chemicals as well as effective dose fifty ($ED_{50}$) values of clinically used antibiotics in silkworm model were found to be similar to those in mammalian models (Hossain et al., 2006; Hamamoto et al., 2004; Hamamoto et al., 2009). In addition, drugs clinically effective only on parenteral administration were effective on intrahemolymph injection but did not show any effect on oral administration in silkworm suggesting that the pharmacokinetics in silkworm and mammals are similar (Hamamoto et al., 2005). Similarly, the metabolic pathway of 4-methyl umbelliferone and 7-ethoxycoumarin were found to be common in silkworm and mammals (Hamamoto et al., 2009). It suggests that the basic metabolic pathway is similar between silkworm and mammals.

In this study, I have used silkworm infection model to screen for therapeutically effective antimicrobial agents and was able to identify two novel compounds from natural as well as chemical library of synthetic compounds.

## Results and discussion

### *a. Kaikosin E*

#### Identification of kaikosin E from soil dwelling bacteria

A total of 15580 bacteria were isolated from soil of Japan and screened for *in-vitro* activity in methicillin susceptible *Staphylococcus aureus* (MSSA). Out of them, 3525 (23%) of the bacteria were found to produce anti-staphylococcal antibiotic *in-vitro*, however, only 23

(0.1%) showed therapeutic activity in silkworm infection model infected with MSSA **(Table 1).** Among the bacteria that showed therapeutic activity in silkworm infection model, a potential new lysobacter strain (2180-5) was selected for further experiments. The culture broth of this strain was also found to be effective against two clinical isolates of methicillin resistant *Staphylococcus aureus* (MRSA). The antibiotic produced by the lysobacter strain was purified and identified as a novel antibiotic during my previous research work in my master's degree. The summary of the purification is shown in **Table 2.** The increase in specific activity is 300 fold. The decrease of the *in-vitro* minimum inhibitory concentration (MIC) values from initial fraction to the final fraction is 1/5 fold while that of therapeutic activity in silkworm model ($ED_{50}$) value is 1/300 fold. This result suggests that chasing after therapeutic activity could identify this antibiotic, which would have been ignored or missed if *in-vitro* activity was chased for purification.

**Structure elucidation**

The structure was elucidated by MS/MS and NMR analyses as shown in **Figure 1a, 1b, 1c and 1d.** Kaikosin E was found to be a cyclic lipopeptide containing 12 amino acid residues and short fatty acid chain with a molecular mass of 1617. It was found to be a novel antibiotic and was named kaikosin after silkworm (Japanese: kaiko) since it was purified using silkworm infection model.

**Antimicrobial activity**

Kaikosin E was found to be effective against Gram-positive bacteria including methicillin resistant strains and mycobacteria with MIC values ranging from 0.5-100 μg $ml^{-1}$ **(Table 3).** It

did not show any activity against Gram-negative bacteria and fungi. Thus, it is a narrow spectrum antibiotic which might be a plus point as the therapy of infectious diseases is now switching from initial use of broad spectrum to narrow spectrum antibiotics, where applicable in order to reduce the occurrence of resistant strains and specifically treat infections without endangering the host immune system.

**Thermostability**

Kaikosin E was found to be thermo-stable as its activity did not change after autoclaving it at 121°C for 20 minutes **(Table 4).**

**Activity in mouse infection model**

Kaikosin E was found to be more effective with $ED_{50}$ value of 0.6 mg $kg^{-1}$ than vancomycin ($ED_{50}$: 1.6 mg $kg^{-1}$) as shown in **Table 5**. Toxicity test on mouse showed that its acute toxic dose is more than 400 mg kg-$^{1}$, i.e, the ratio of $LD_{50}/ED_{50}$ is more than 600. This result suggests that kaikosin E has high efficacy and low toxicity having wide therapeutic index, which makes it suitable candidate for further clinical studies. Usually, drugs that have the ratio of $LD_{50}/ED_{50}$ more than 100 are considered safe, and the larger the ratio, the safer the drug. In this regard, kaikosin E is much safer.

**Killing/Bactericidal effect**

Killing assay was performed to determine the mode of action of kaikosin E by counting the colony forming unit per milliliter (CFU $ml^{-1}$) of bacteria at different time intervals after treatment with antibiotics. Kaikosin E was found to have a potent and rapid killing effect to

exponentially growing *S. aureus* **(Table 6).** The killing ability was so rapid that more than 99% of bacteria were killed within one minute of exposure of kaikosin E and its inhibitory concentration fifty ($IC_{50}$) value was calculated as 0.4 µg $ml^{-1}$, far less than its MIC value **(Figure 2).** The potent and rapid bactericidal activity even at concentrations lower than MIC value is a unique property of kaikosin E which triggered the hypothesis that its antibacterial mechanism of action might be different than the existing known mechanisms of known antimicrobial agents.

**Bacteriolytic activity**

To test if kaikosin E lyses bacterial cells, this experiment was performed. Kaikosin E was found to have bacteriolytic activity potent than that of vancomycin and daptomycin as shown in **Figure 3.**

**Inhibition of macromolecule biosynthesis**

Kaikosin E inhibited the incorporation of radiolabeled thymidine, uridine, methionine, and N-acetylglucosamine suggesting the inhibition of DNA, RNA, protein and peptidoglycan respectively **(Figure 4 a, b, c, d).** The rapid inhibition of all macromolecule biosynthesis in *S. aureus* led to the hypothesis that this antibiotic can damage the bacterial membrane thus rendering a halt to biosynthesis of macromolecules.

**Dissipation of membrane potential**

The rapid bactericidal and bacteriolytic activity and inhibition of the macromolecule biosynthesis led to the hypothesis that kaikosin E damages the bacterial membrane. To test

this hypothesis, dissipation of membrane potential was measured using a membrane potential sensitive flourscent dye $DisC_3(5)$. $DiSC_3(5)$ self-quenches its own fluorescence inside cell membrane according to membrane potential. Compound that dissipates the membrane potential causes release of the dye with consequent increase in fluorescence. Therefore, the increase in flourscence is directly related to the increase in dye release which in turn is related to the dissipation of membrane potential. The addition of kaikosin E rapidly dissipated the membrane potential in *S. aureus*. This dissipation was stronger than that of pore forming antibiotic Nisin. Kaikosin E dissipated the membrane potential at a concentration of 0.05 μg $ml^{-1}$, which is far lower than its MIC value (**Figure 5)**. This result suggested the rapid bacterial membrane damage by kaikosin E.

| | |
|---|---|
| Separated bacteria | 15580 |
| Antimicrobial activity | 3525 (23%) |
| Therapeutic activity | 23 (0.1%) |

**Table 1: Screening of therapeutically effective samples from soil bacteria**. Bacteria were separated from soil of Japan on agar plates and culture broth of these bacteria were screened for antimicrobial activity against MSSA using broth dilution assay. The positive samples were subjected to therapeutic effect evaluation using silkworm bacterial infection model infected with MSSA.

| Fraction | Total activity (units) | Recovery (%) | Dry weight (mg) | Specific activity (units $mg^{-1}$) | $ED_{50}$ (µg $g \cdot larva^{-1}$) | MIC (µg $ml^{-1}$) |
|---|---|---|---|---|---|---|
| Acetone extract | 180000 | 100 | 16000 | 11 | 90 | 25 |
| Butanol extract | 160000 | 89 | 646 | 250 | 4 | 0.6 |
| Water precipitation | 41000 | 23 | 75 | 550 | 1.8 | N.D. |
| ODS column chromagtoraphy | 44000 | 25 | 22 | 2000 | 0.5 | N.D. |
| RP-HPLC P5 | 17000 | 10 | 5.0 | 3400 | 0.3 | 5 |

*N.D.: Not Determined

**Table 2: Purification summary of kaikosin E.** Kaikosin E was purified by organic solvent extraction followed by octadecylsilyl (ODS) open column chromatography and reverse phase high performance liquid chromatography (HPLC). Each fraction was tested for *in-vitro* antimicrobial activity and therapeutic activity in silkworm infection model. Kaikosin E was separated as P5.

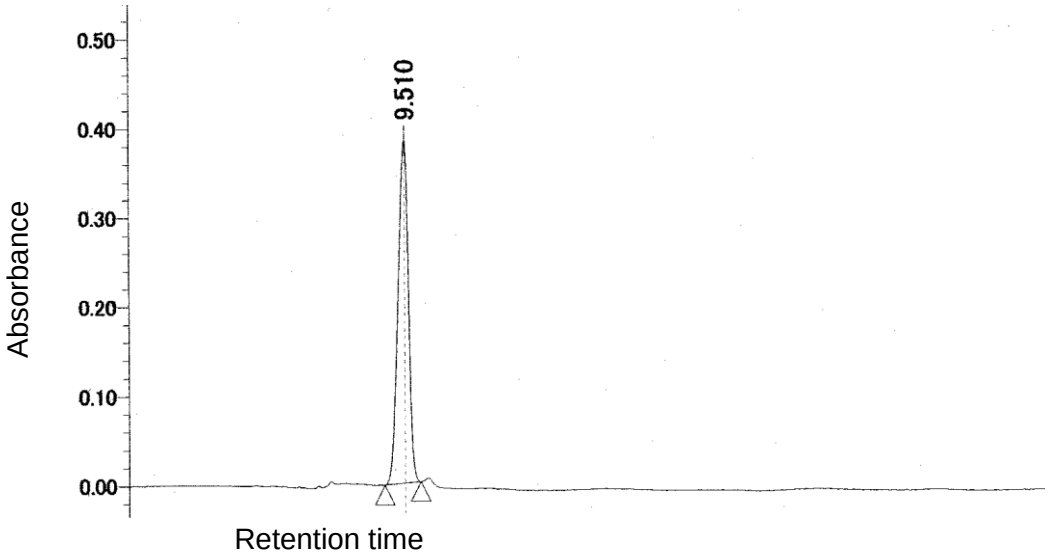



**Figure 1 a: HPLC peak pattern of kaikosin E**. The final fraction obtained was injected to RP-HPLC analytical column (Senshu Pak Pegasil ODS SP100, 4.6 ϕ x 250 mm) equilibrated with 75-95% methanol containing 0.1% trifluoroacetic acid.

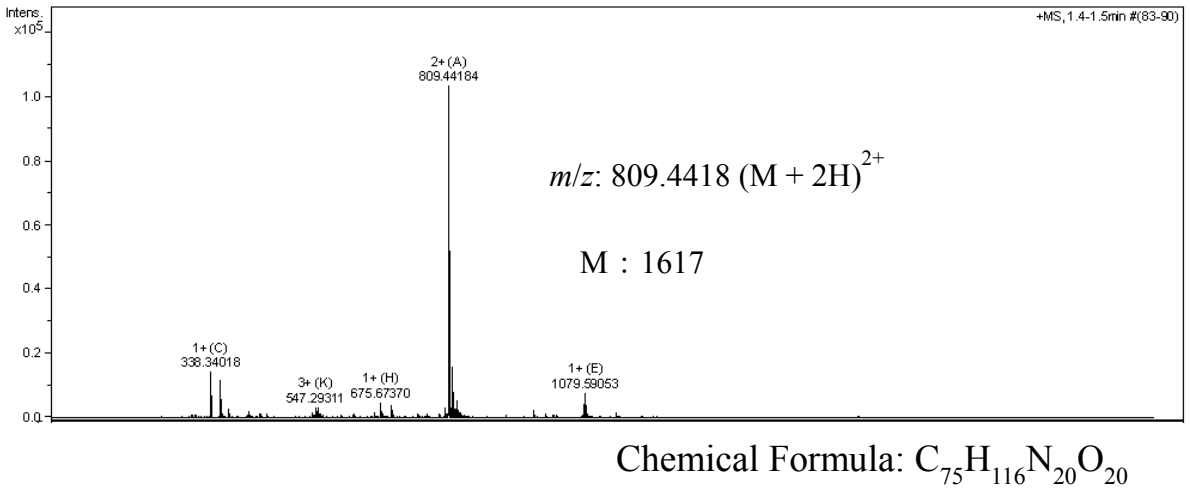



**Figure 1 b: Mass spectrometric analysis of kaikosin E.** Kaikosin E was applied to liquid chromatography-mass spectrometer (LC-MS) and molecular mass was calculated.

$^{1}H$

$^{13}C$

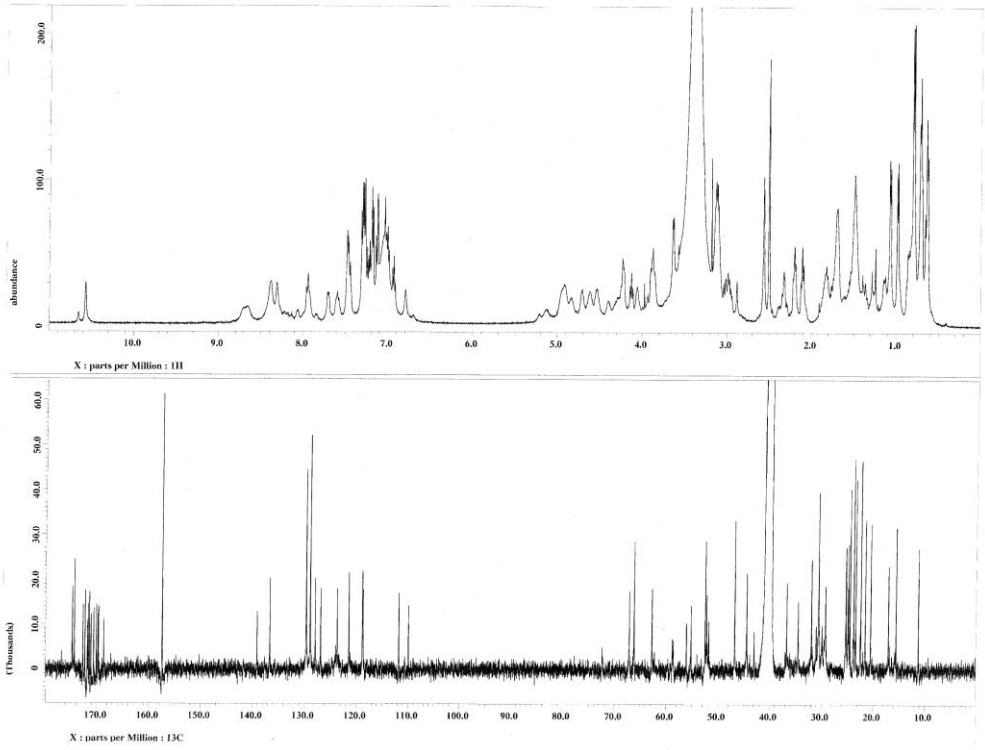



**Figure 1 c: Proton and carbon nuclear magnetic resonance (NMR) analyses of kaikosin E**

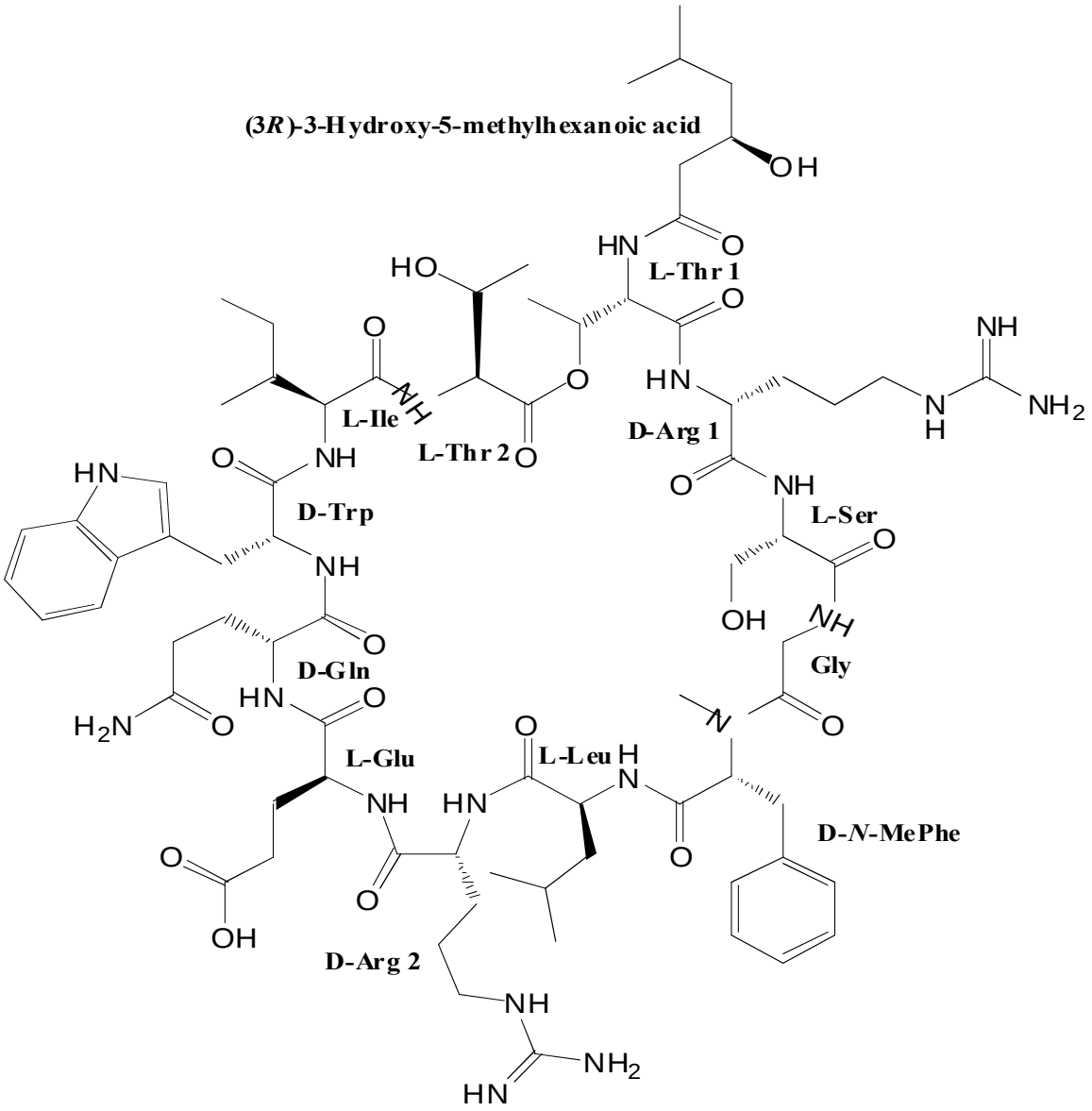



**Figure 1d: Structure of kaikosin E**

**Figure 1: Structural elucidation of kaikosin E**

| Microorganism | MIC (μg ml$^{-1}$) |
|---|---|
| Methicillin-susceptible *S. aureus* MSSA1 | 4 |
| Methicillin-resistant *S. aureus* MRSA3 | 4 |
| Methicillin-resistant *S. aureus* MRSA4 | 4 |
| *Staphylococcus aureus* Smith | 4 |
| *Bacillus subtilis* | 2 |
| *Bacillus cereus* | 2 |
| *Listeria monocytogenes* | 0.5 |
| *Streptococcus pneumonia* | 25 |
| *Streptococcus sanguinis* | 100 |
| *Streptococcus pyogenes* | 50 |
| *Streptococcus agalactiae* | 100 |
| *Mycobacterium fortuitum* | 8 |
| *Mycobacterium smegmatis* | 8 |
| *Salmonella enterica* | >100 |
| *Serratia marcescens* | >100 |
| *Psedomonas aeruginosa* PA01 | >100 |
| *Escherichia coli* W3110 | >100 |
| *Candida albicans* | >100 |
| *Candida tropicalis* | >100 |
| *Cryptococcus neoformans* | >100 |

**Table 3: Antimicrobial spectrum of kaikosin E.** Antimicrobial activity of kaikosin E was determined against different microorganisms with broth dilution assay.

| Sample | MIC (μg $ml^{-1}$) |
|---|---|
| Kaikosin E autoclaved | 3.2 |
| Kaikosin E untreated | 3.2 |

**Table 4: Thermo-stability of kaikosin E**. Minimum inhibitory concentration of kaikosin E was determined by broth dilution assay with and without heat treatment to kaikosin E.

| **Antibiotics** | **$ED_{50}$ (mg $kg^{-1}$)** |
|---|---|
| Kaikosin E | 0.6 |
| Vancomycin | 1.6 |

**Table 5: Therapeutic activity in mouse sepsis model.** Mice were infected with *S. aureus* Smith followed by injection of antibiotics after 1 hour. The survival of mice was counted after 2 days.

| Time (min) | CFU $ml^{-1}$ of *S. aureus* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Kaikosin E (µg $ml^{-1}$) | | | | | | Daptomycin | No drug |
| | 1.25 | 2.5 | 5 | 10 | 20 | 25 | 5µg $ml^{-1}$ | |
| 1 | 3 x$10^5$ | 2 x$10^5$ | 3 x$10^5$ | <1 x$10^3$ | <1 x$10^3$ | <1 x$10^3$ | 2.6 x$10^7$ | 2.0 x$10^7$ |
| 15 | 1 x$10^5$ | 8 x$10^4$ | 1 x$10^5$ | <1 x$10^3$ | <1 x$10^3$ | <1 x$10^3$ | 7.6x$10^5$ | 6.0 x$10^7$ |
| 30 | 4 x$10^4$ | 4 x$10^4$ | 4 x$10^4$ | <1 x$10^3$ | <1 x$10^4$ | <1 x$10^3$ | 4.0x$10^4$ | 4.0 x$10^7$ |
| 60 | 4 x$10^4$ | <1 x$10^4$ | 4 x$10^4$ | <1 x$10^3$ | <1 x$10^3$ | <1 x$10^3$ | <1.0x$10^4$ | 2.0 x$10^8$ |
| 120 | 7 x$10^4$ | <1 x$10^4$ | 7 x$10^4$ | <1 x$10^3$ | <1 x$10^3$ | <1 x$10^3$ | 1.0x$10^4$ | 8.6 x$10^8$ |

**Table 6: CFU $ml^{-1}$ of *S. aureus* after drug treatment.** Colony forming unit per milliliter of bacteria was counted at different time intervals after treatment with different antibiotics by diluting and spreading the aliquots on agar plates and incubating for 24 hours.

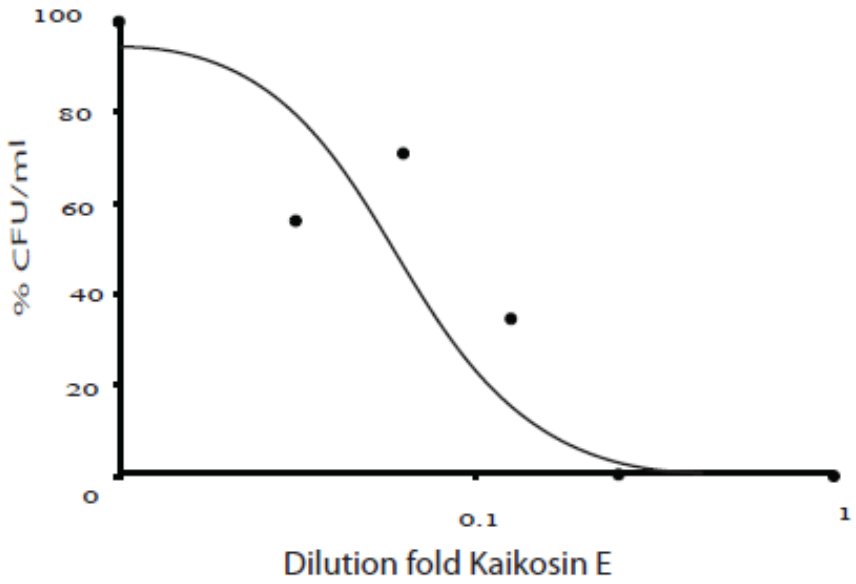



**Figure 2: $IC_{50}$ of killing effect of kaikosin E.** $IC_{50}$ value was calculated based on the CFU $ml^{-1}$ of bacteria at 60 minutes after treatment with different concentrations of kaikosin E.

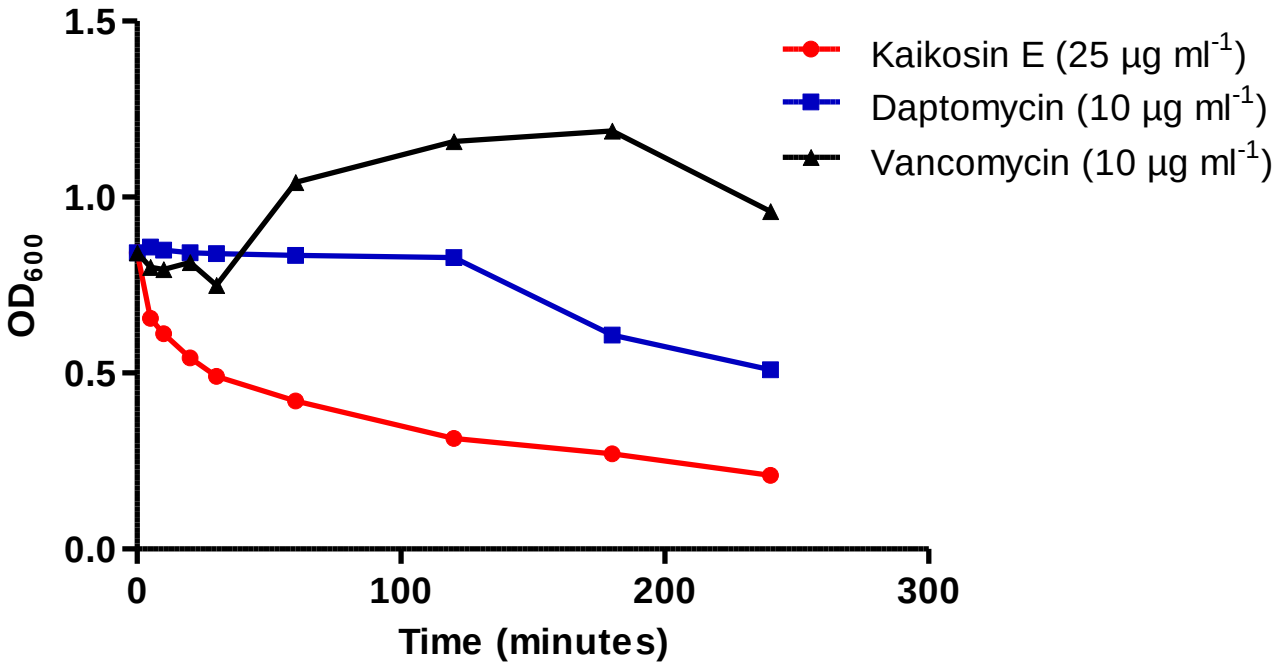
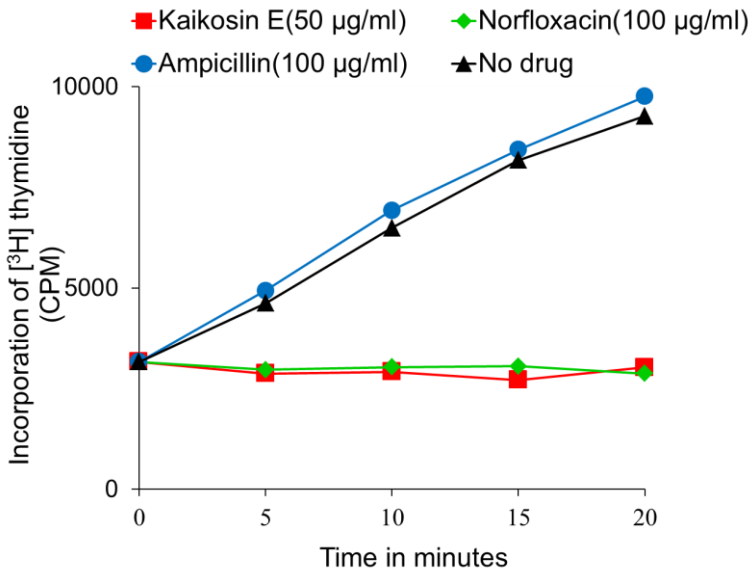
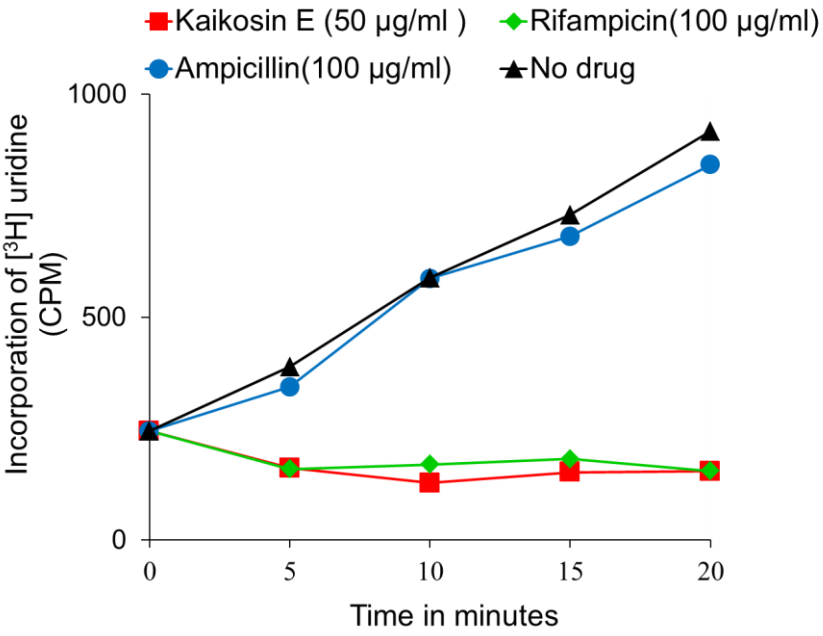


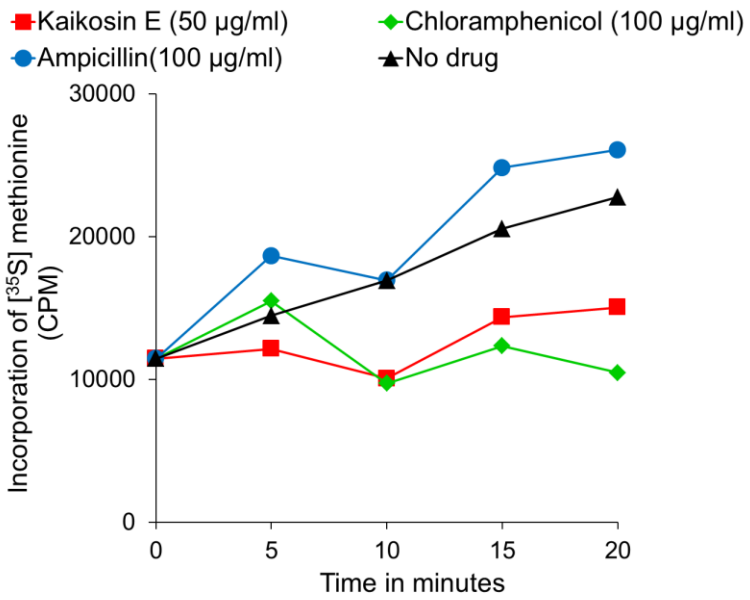

**Figure 3: Bacteriolytic activity of kaikosin E.** Exponentially growing *S. aureus* was treated with different antibiotics, daptomycin, vancomycin and kaikosin E. Aliquots were sampled at different time intervals as indicated and the optical density at wavelength 600 nm was measured.



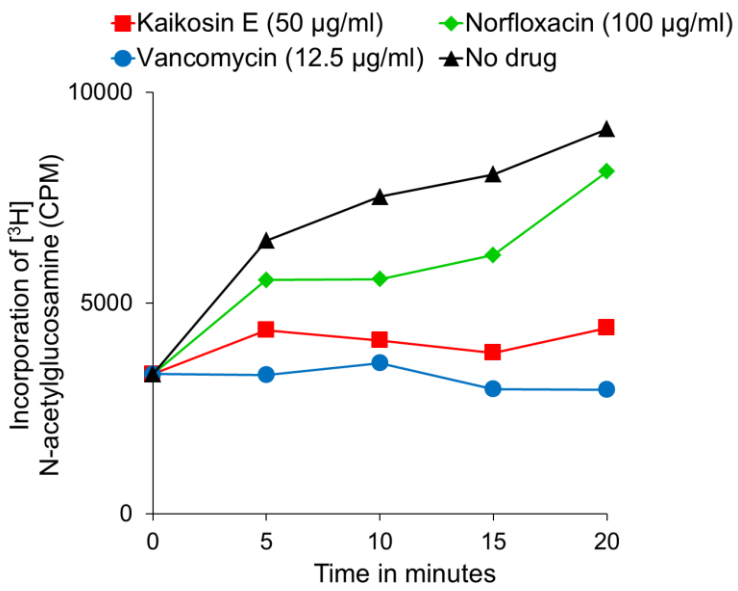

**a. DNA synthesis inhibition**




**b. RNA synthesis inhibition**




**c. Protein synthesis inhibition**




**d. Peptidoglycan synthesis inhibition**

**Figure 4: Inhibition of macromolecule biosynthesis.** Radiolabeled precursors were added to expontentially growing *S. aureus* in presence or absence of antibiotics. Samples were collected at different time intervals as indicated and radioactivity of acid insoluble fractions were measured by liquid scintillation counter

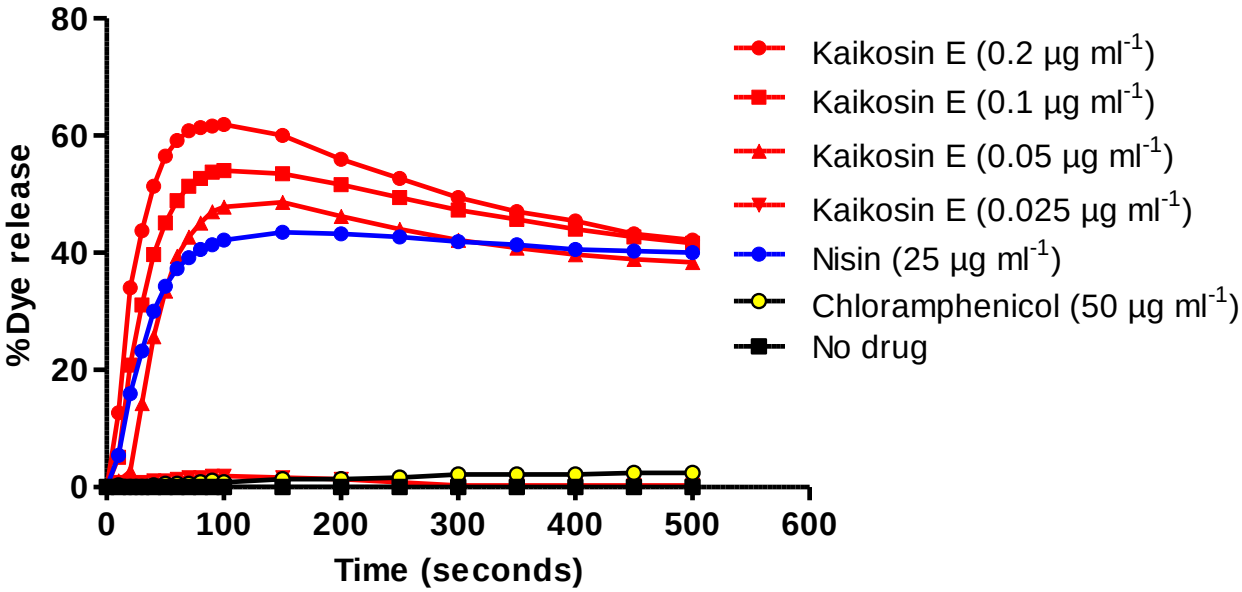



**Figure 5: Dissipation of membrane potential.** Exponentially growing bacterial cells were treated with the membrane potential sensitive dye $DiSC_3(5)$ and then with different antibiotics as indicated. Aliquots (2 ml) were transferred to a polystyrene fluorimeter cuvette containing a stir bar and placed in the heated (37°C) sample chamber of a spectrofluorometer. The cells were excited at 622 nm, and the fluorescence emission was collected at 670 nm.

**b. Compound 363**

**Identification**

A total of 103,873 synthetic compounds were screened for *in-vitro* activity against MSSA. Out of them, 3,383 (3%) showed *in-vitro* activity and only 3 (0.003%) showed therapeutic activity in silkworm infection model **(Table 7).** Among the compounds that showed therapeutic activity in silkworm infection model, compound 363 **(Figure 6)** was selected for further study as it was the most potent compound.

**Antimicrobial activity**

Compound 363 was found to be effective mainly against *S. aureus* including methicillin resistant strains with MIC value of 6.3 µg $ml^{-1}$ **(Table 8).** This shows specificity of compound 363 to *S. aureus* which is sometimes a desired property in clinical practice.

**Activity in silkworm infection model**

Compound 363 exerted therapeutic activity in silkworm infection model with an $ED_{50}$ value of 39 µg g•$larva^{-1}$ **(Figure 7)**

**Activity in mouse infection model**

Mice infected with *S. aureus* survived when given 100 mg $kg^{-1}$ of compound 363 **(Figure 8)** suggesting that compound 363 is therapeutically effective in mouse infection model too.

**Killing/Bactericidal effect**

Killing assay was performed to know the mode of action of compound 363. Usually more than or equal to 3 log decrease in the initial CFU $ml^{-1}$ is defined as the bactericidal effect and

less than that is considered bacteriostatic effect. The CFU $ml^{-1}$ of bacteria did not change after 24 hours of treatment with compound 363 suggesting that it exerted bacteriostatic activity **(Figure 9).**

**Inhibition of macromolecule biosynthesis**

Compound 363 inhibited incorporation of [$^{3}$H]uridine and slightly inhibited the incorporation of [$^{3}$H]thymidine while did not inhibit the incorporations of [$^{35}$S]methionine and [$^{3}$H]N-acetylglucosamine **(Figure 10 a, b, c, d).** These results suggested that it inhibited RNA synthesis, with slight inhibition of DNA synthesis.

| | |
|---|---|
| Tested compounds | 103,873 |
| Antimicrobial activity | 3,383 (3%) |
| Therapeutic effectiveness | 3 (0.003%) |

**Table 7: Screening of therapeutically effective chemical compounds using silkworm infection model**. Library of synthetic chemical compounds were screened for *in-vitro* activity against MSSA and the positive samples were further tested for therapeutic activity using silkworm infection model infected with MSSA

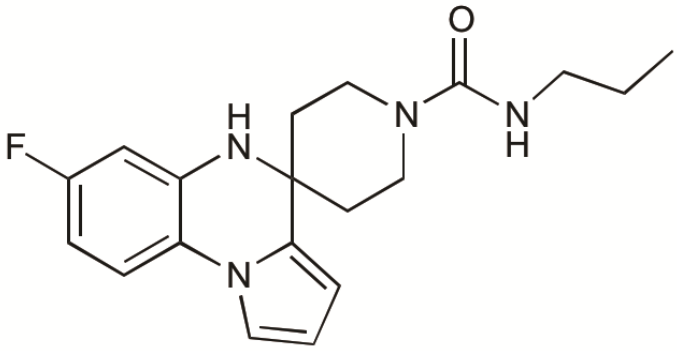



**Figure 6: Compound 363:** Chemical structure of compound 363

| **Microorganism** | **MIC (μg ml$^{-1}$)** |
|---|---|
| *Staphylococcus aureus* RN4220 | 12.5 |
| Methicillin-susceptible *S. Aureus* MSSA1 | 6.3 |
| Methicillin-resistant *S. aureus* MRSA4 | 6.3 |
| *Enterococcus faecalis* EF1 | >400 |
| Vancomycin-resistant *Enterococcus faecalis* EF5 | >400 |
| *Bacillus subtilis* JCM2499 | 200 |
| *Bacillus cereus* JCM20037 | 100 |
| *Listeria monocytogenes* | 100 |
| *Streptococcus pneumonia* | 100 |
| *Streptococcus agalactiae* JCM5671 | >400 |
| *Streptococcus sanguinis* JCM5678 | >400 |
| *Streptococcus pyogenes* | 200 |
| *Escherichia coli* | >400 |
| *Pseudomonas aeruginosa* PAO1 | >400 |

**Table 8: Antimicrobial spectrum of compound 363.** Antimicrobial activity of compound 363 was determined by broth dilution assay against different microorganisms.

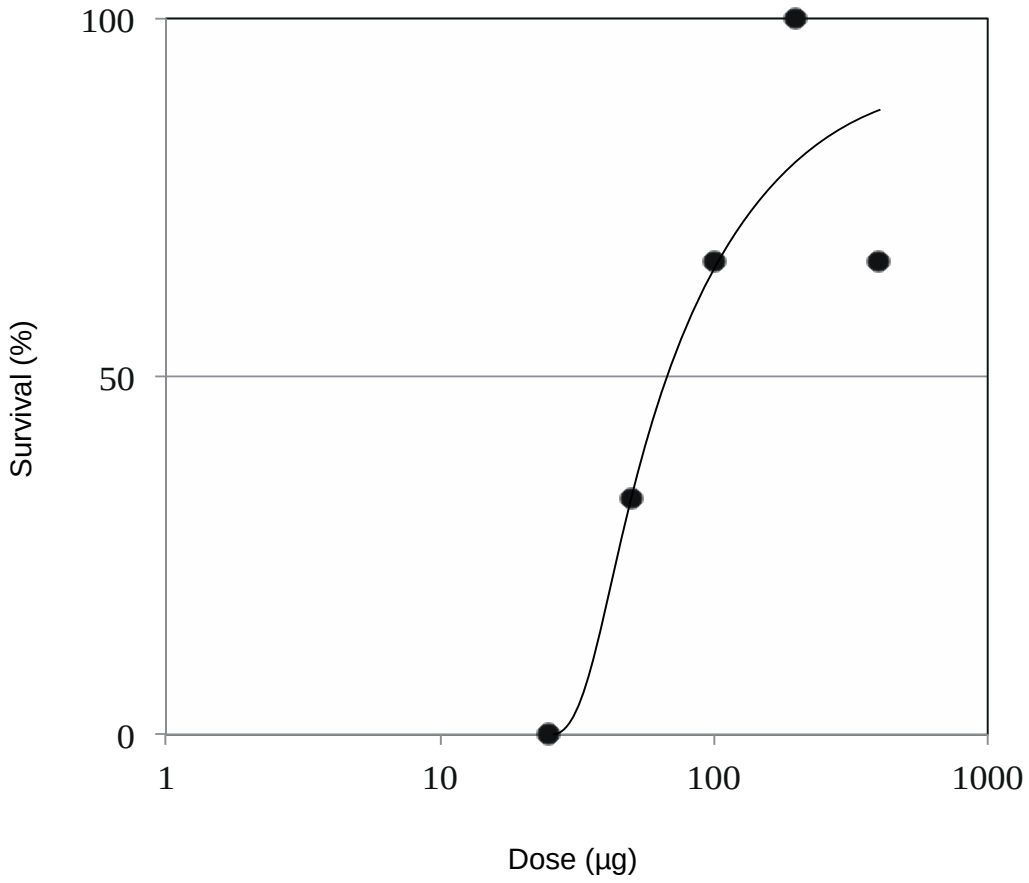



**Figure 7: Therapeutic activity of compound 363 in silkworm infection model.** Silkworms infected with *S. aureus* were injected with compound 363 at different doses and survival of silkworm was observed after 2 days. The $ED_{50}$ value was calculated to be 39 µg g•larva$^{-1}$.

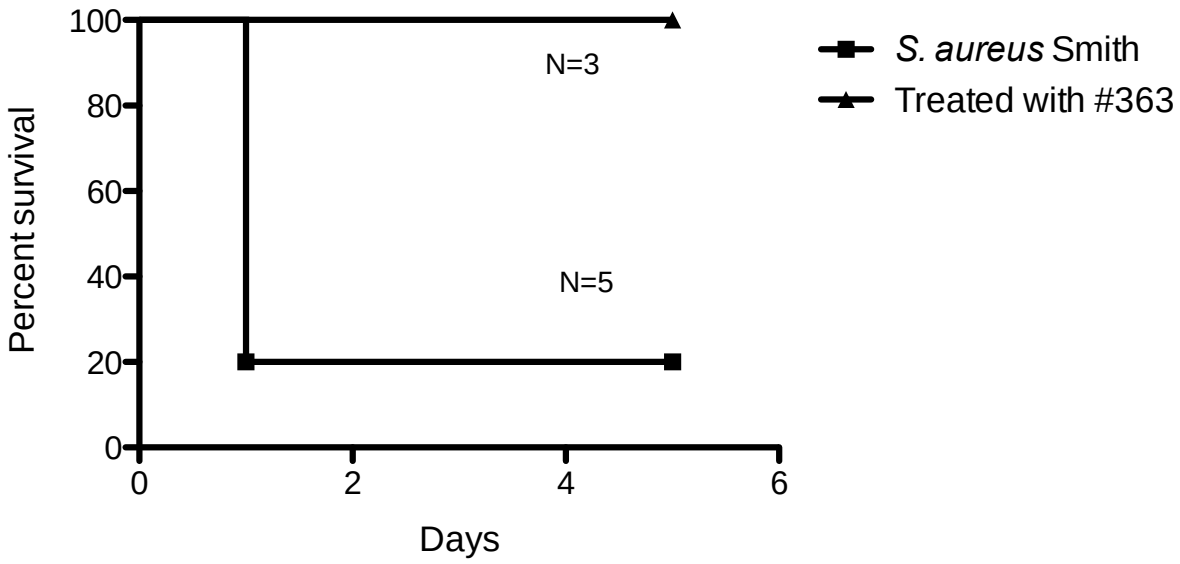



**Figure 8: Therapeutic activity of compound 363 in mouse infection model.** Mice infected with *S. aureus* Smith were given 100 mg $kg^{-1}$ of compound 363 intraperitoneally and observed for several days.

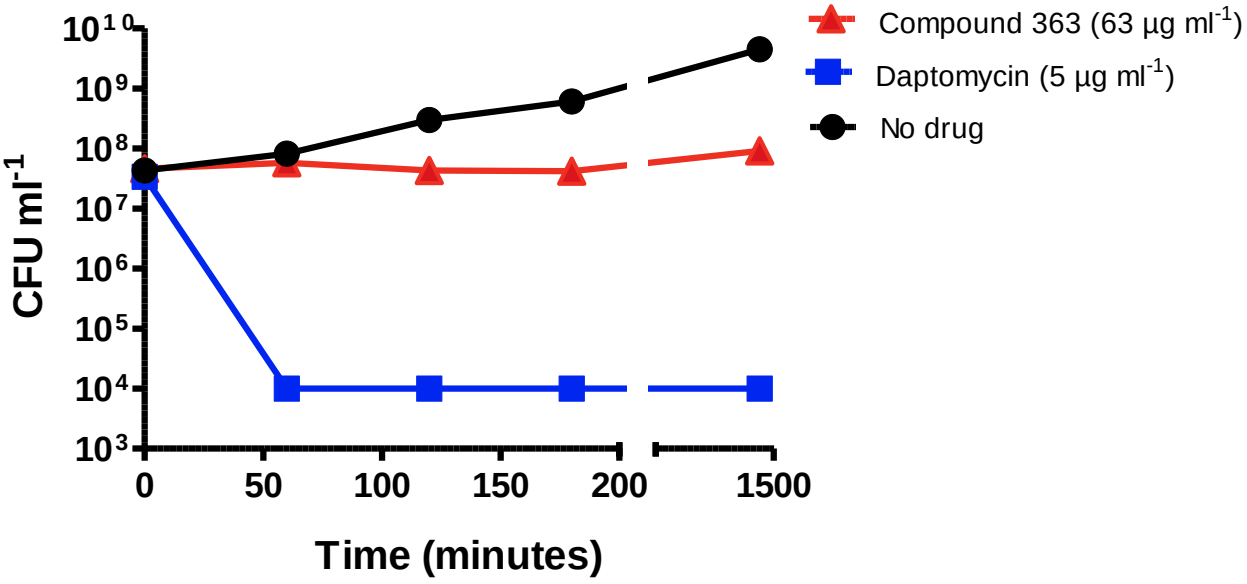
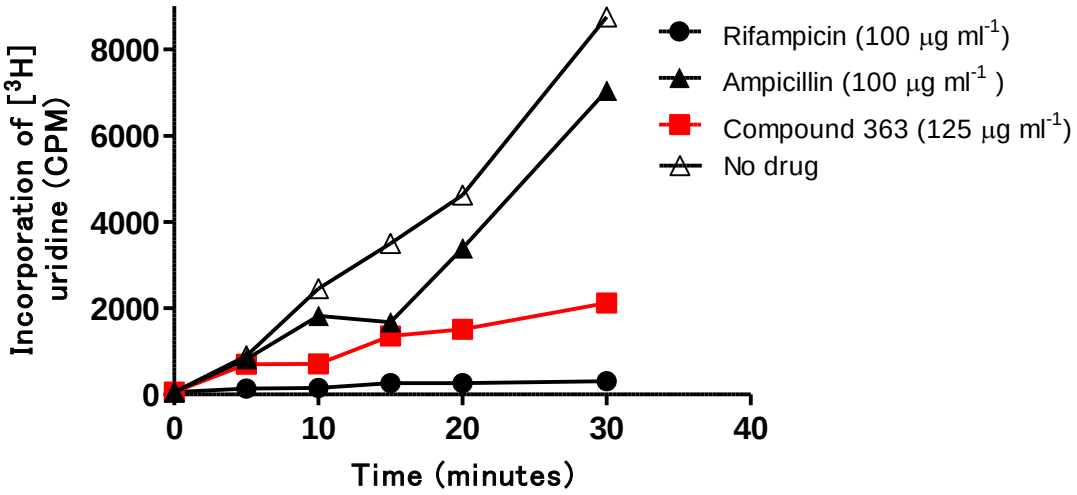
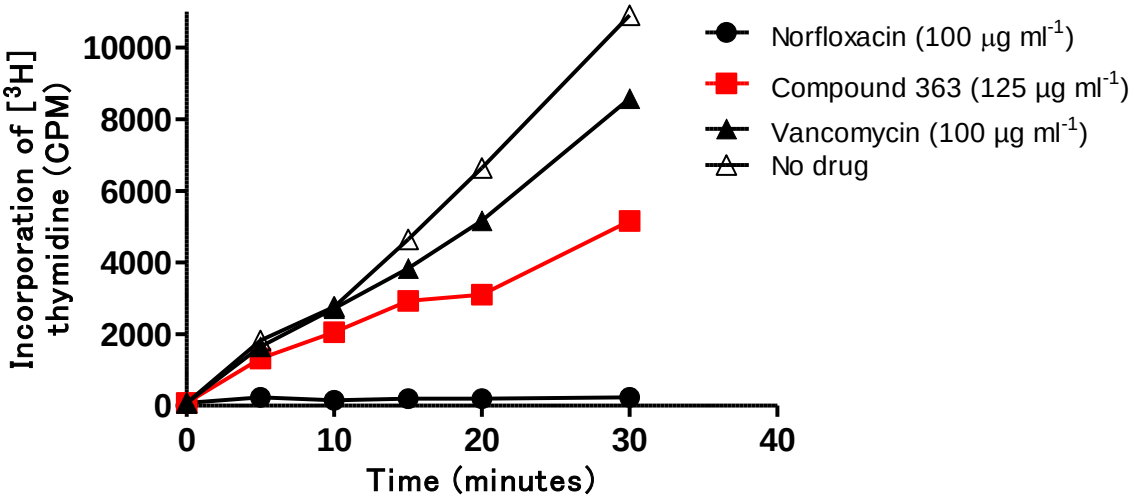



**Figure 9: Bacteriostatic activity of compound 363**

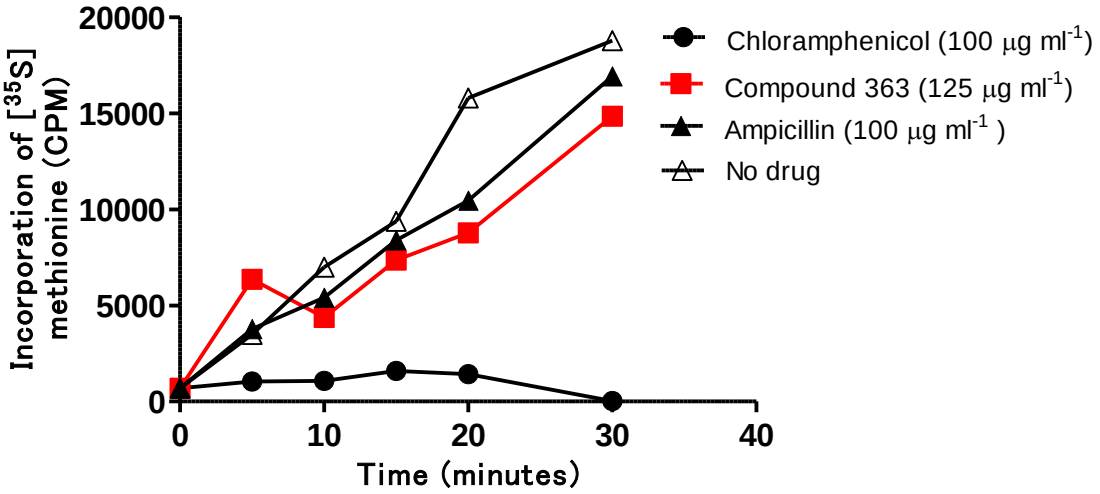
Exponentially growing *S. aureus* was treated with antibiotics. Culture aliquots were collected at different time as shown, diluted and spread on agar plates and incubated at 37°C for 24 hours. Cell viability was determined by counting the CFU $ml^{-1}$.



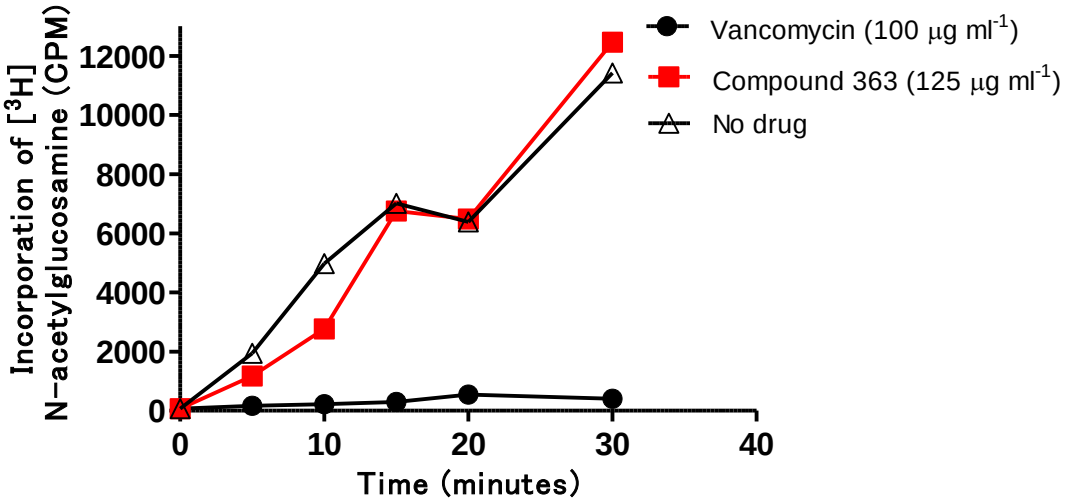

**a. Effect on RNA synthesis**




**b. Effect on DNA synthesis**




**c. Effect on protein synthesis**




**d. Effect on peptidoglycan synthesis**

**Figure 10: Effect of compound 363 on macromolecule biosynthesis.** Radiolabeled precursors were added to expontentially growing *S. aureus* in presence or absence of antibiotics. Samples were collected at different time intervals as indicated and radioactivity of acid insoluble fractions were measured by liquid scintillation counter.

## Conclusion

Two novel therapeutically effective antimicrobial agents; kaikosin E and compound 363 were identified using silkworm bacterial infection model. Therefore, in the era where conventional screening systems for antimicrobial agents have become almost outdated and ethically acceptable new systems that improve selectivity are desirable, silkworm infection model can be an effective model for screening therapeutically effective compounds and useful in discovery of novel antimicrobial agents.

**PART 2**

**Identification of cellular target of the antimicrobial agents in *S. aureus***

**Background**

Whenever a new compound is identified that is therapeutically effective and has a potential to be used for clinical purposes, the elucidation of mechanism becomes important. The elucidation of mechanism of action of such compounds not only helps in understanding the pharmacokinetics, pharmacodynamics and fate of drug molecules, but also helps in reducing treatment failure. Many compounds enter the costly clinical trials without their mechanism being elucidated and face failures to proceed forward in the trials. Only few drugs are approved by the Food and Drug Administration of the US every year with fewer drugs of known mechanism of action (Drews, 2003). One of the possible explanations for the drugs to face failures during the clinical trials is due to the poorly understood mechanism of action. For the approved drugs whose mechanism is not well known, failures may cause serious damage to public health. Therefore, identifying cellular target of novel compounds is very important. The mechanistic analysis also helps in drug development by allowing adequate modification. The modification can be for improving the drug-target binding. Hence, a single target can provide a wide array of effective agents.

**Temperature Sensitive (TS) mutants screening**

One of the strategies to find the drug target is to identify the gene(s) responsible for resistance by isolating drug resistant strains. However, this has certain limitations because all the genes responsible for resistance not necessarily are related to the target; sometimes they are related to only the resistance mechanism like the efflux pump or some transporters. Another problem arises when the level of resistance is not high enough to evaluate and cannot be excluded within the experimental error which gives false results. Hence, in such

cases, if the focus can be made on mutation in essential genes in the resistant strains, the chances of getting closer to the drug target is higher. For such a purpose, TS screening is a helpful tool as mutations in essential genes correlate to TS phenotype. Usually, the mutations conferred are point mutations leading to change in an amino acid sequence of a protein. The stability of protein becomes dependent upon the temperature and the protein cannot function at higher temperatures but can still function at lower temperatures. Since drugs target essential component of bacteria to exert activity and TS screening helps to identify mutations in essential genes, if the gene responsible for TS phenotype is identified, the chances of this gene being related to the target are higher. With this point in consideration, I have used TS screening strategy to identify the cellular target of kaikosin E.

**Results and discussion**

**a. Cellular target of kaikosin E in *S. aureus***

**Kaikosin E resistant strains and screen for TS phenotype**

A total of 105 strains of mutagen ethylmethylsulfonate (EMS) treated *S. aureus* strain RN4220 were isolated from kaikosin E containing agar plates. Among 105 kaikosin E resistant strains, two colonies showed temperature sensitive phenotype: #5 and #9 that were obtained from 5 μg $ml^{-1}$ kaikosin E containing agar plate **(Figure 11)**. The MIC value of kaikosin E for these strains was found to be 2 fold higher than that for the parent strain RN4220 **(Table 9).** Thus, the level of resistance to kaikosin E was low in the mutated strains. For such cases, the analysis of gene responsible for resistance would have been difficult that was overcome here by the use of TS strategy.

**Mutated genes**

Upon DNA library insertion and sequence analysis, *menA* (SA0894) and *fni* (SA1236) genes were identified to have mutations in #5 and #9 TS mutants respectively. Sequencing analysis revealed the mutation points as shown in **Table 9.**

***menA* and *fni* complemented TS phenotype**

When wild type *menA* and *fni* genes were inserted into the corresponding mutants, they could grow at both 30°C and 43°C, complementing the TS phenotype **(Figure 12).** This result suggests that *menA* and *fni* genes are responsible for the TS phenotype in the corresponding mutants.

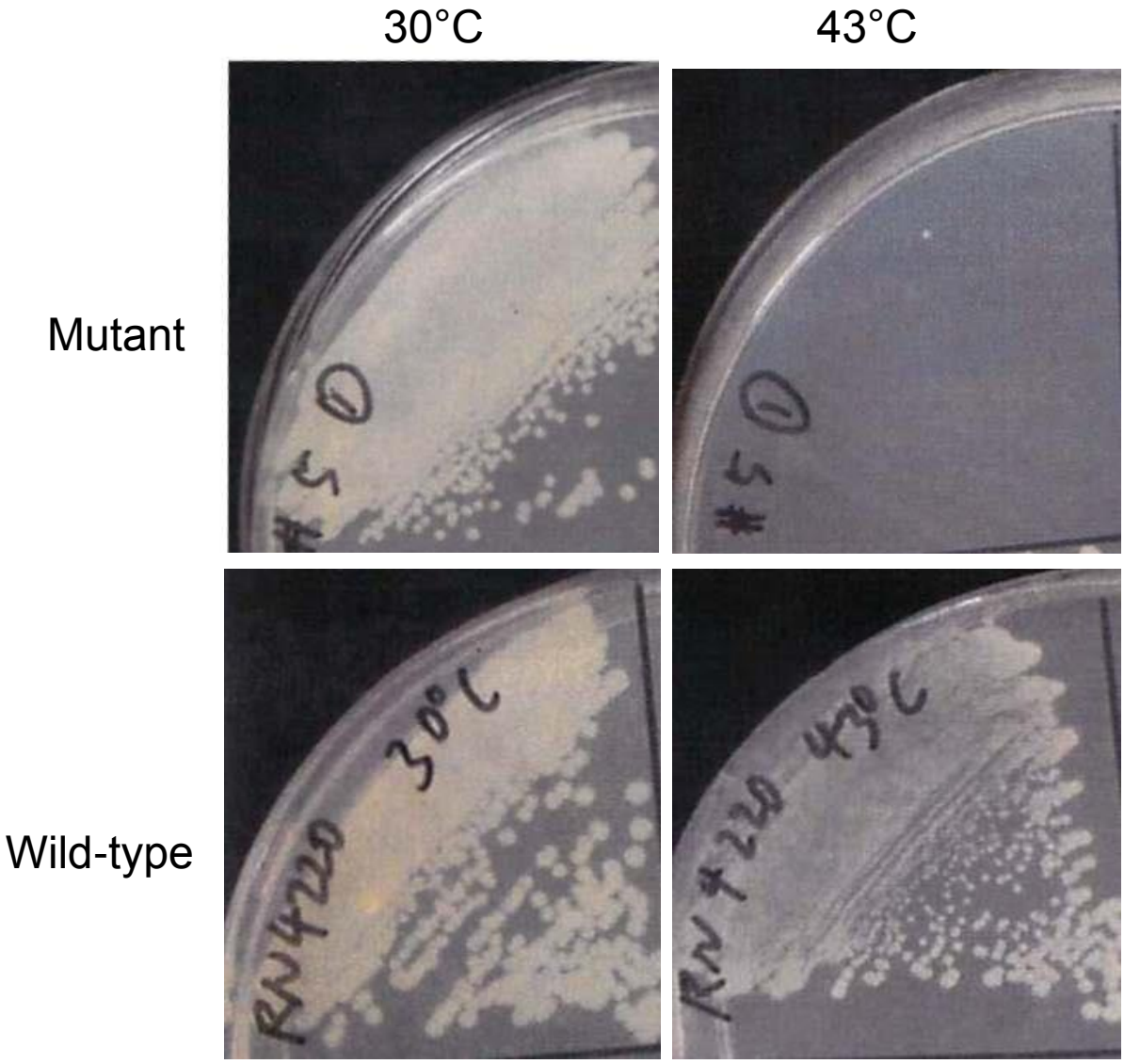
These genes are the essential genes for viability of *S. aureus. menA* gene encodes for 1,4-dihydroxy-2-napthoate (DHNA) octaprenyl transferase, an enzyme responsible for synthesis of demethylmenaquinone (DMK) from DHNA in a biosynthetic pathway whose final product is menaquinone (Bentley and Meganathan,1982). *fni* gene encodes the isopentenyl-pyrophosphate (IPP) isomerase. IPP isomerase is the enzyme that catalyzes the isomerization step essential for biosynthesis of isoprenoids. Isoprenoids and isoprenoid derivatives are required for many essential biosynthetic pathways including menaquinone biosynthetic pathway. Thus, both *menA* and *fni* genes are involved in biosynthetic pathway of menaquinone **(Figure 13)**. Menaquinone is an essential compound required for respiration in *S. aureus.* Mutations in these two genes in kaikosin E resistant strains and involvement of these two genes in menaquinone biosynthetic pathway led to the speculation that kaikosin E might target menaquinone.

**Menaquinone binding assay**

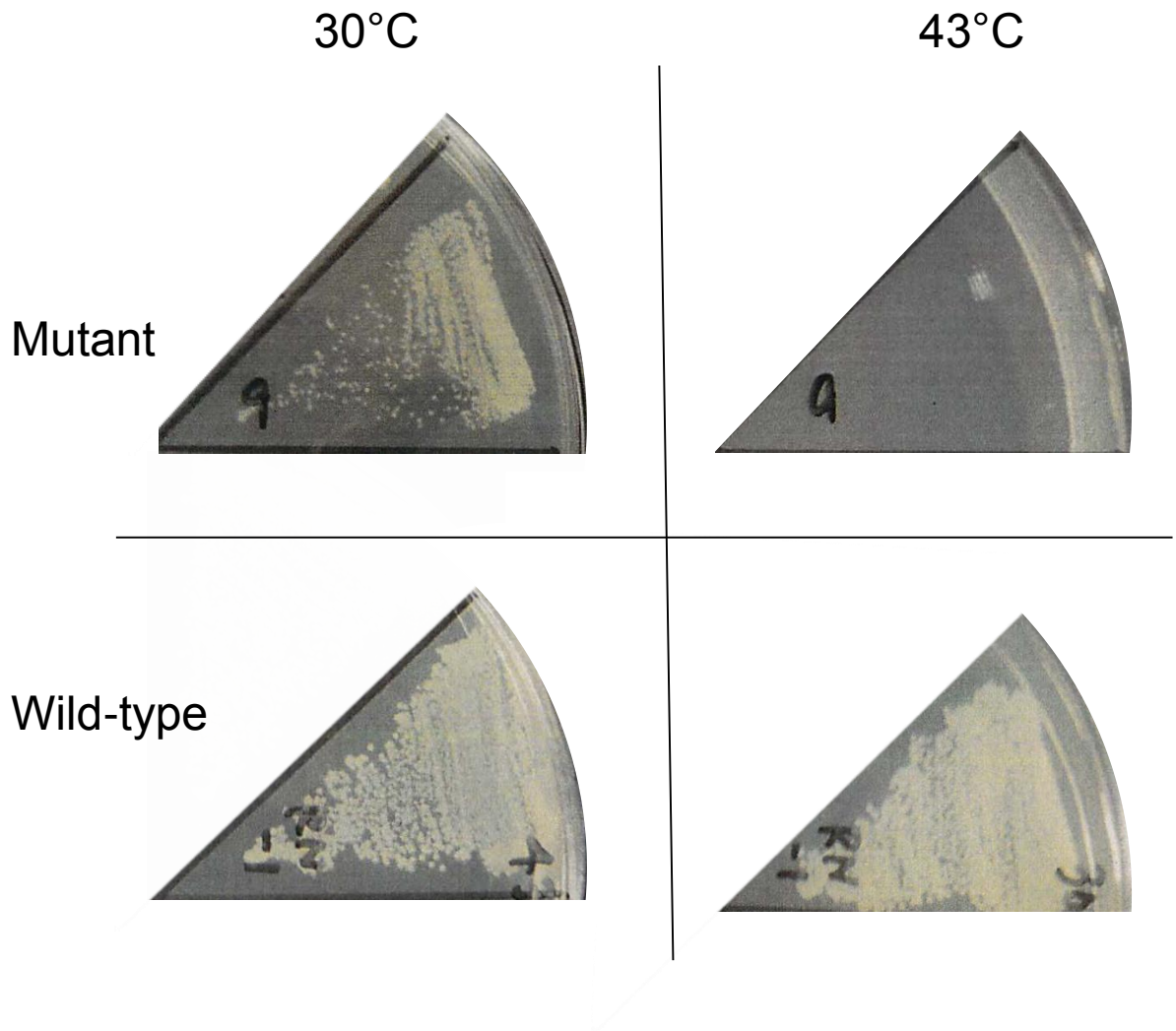
To test the hypothesis if menaquinone is the target of kaikosin E, binding assay was performed. Menaquinone and kaikosin E containing tube showed a clear precipitate after centrifugation while no precipitate was observed in kaikosin E or menaquinone alone tubes **(Figure 14)**. This result clearly suggested that kaikosin E directly binds to menaquinone. Thus, the target of kaikosin E might be menaquinone.

**Effect of menaquinone on antibacterial activity of kaikosin E**

Further to test if menaquinone is the target of kaikosin E, effect of menaquinone addition on the antibacterial activity of kaikosin E was determined. The MIC of kaikosin E against MSSA1 increased with increasing concentration of menaquinone, whereas it did not change with or without ubiquinone **(Figure 15).** This increase in MIC value, i.e., decrease in antibacterial activity, further provided evidence that menaquinone is the target of kaikosin E. Menaquinone has never been reported as target of antibiotics. This is the first report to reveal that menaquinone is target of an antibiotic. Hence, a novel target menaquinone was identified for antimicrobial activity. Moreover, the activity was unaffected upon addition of ubiquinone suggesting that kaikosin E is selectively toxic to bacteria and not toxic to mammals because ubiquinone, but not menaquinone, is required for respiration in mammals. This also explains the non-toxic and safe property of kaikosin E in mouse model.




**a. #5 mutant and wild-type**

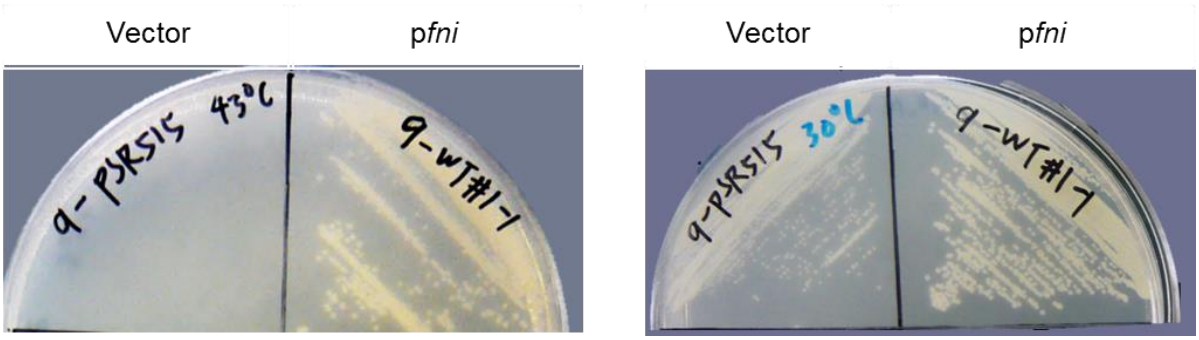



**b. #9 mutant and wild-type**

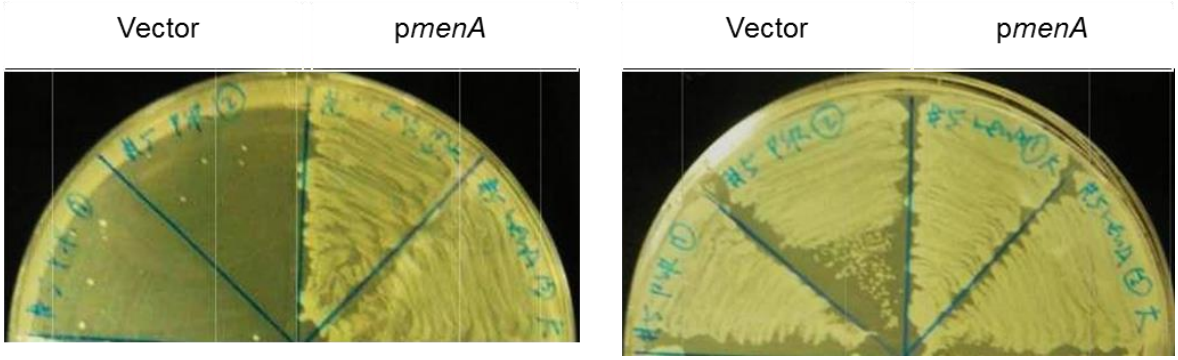
**Figure 11: Kaikosin E resistant TS mutants.** The cells were grown in liquid LB medium overnight, diluted 100 fold with 0.9% NaCl, streaked on NaCl-depleted LB agar plate and incubated at 30°C and 43°C overnight.

| Strain | Mutated gene | Mutation site | MIC (μg $ml^{-1}$) |
|---|---|---|---|
| RN4220 (parent) | | | 3.1 |
| #5 | *menA* (SA0894) | Asp33Gly | 6.3 |
| #9 | *fni* (SA1236) | Glu187Lys | 6.3 |

**Table 9: MIC values of kaikosin E against parent and mutant strains and mutated genes with mutation sites**




**a. *fni* gene**




**b. *menA* gene**

**Figure 12: *fni* and *menA* genes complemented the TS phenotype**

Plasmids harboring wild-type *fni* and *menA* genes were constructed, transformed to the corresponding mutants, streaked on NaCl-depleted LB agar plate and incubated at 30°C and 43°C overnight.

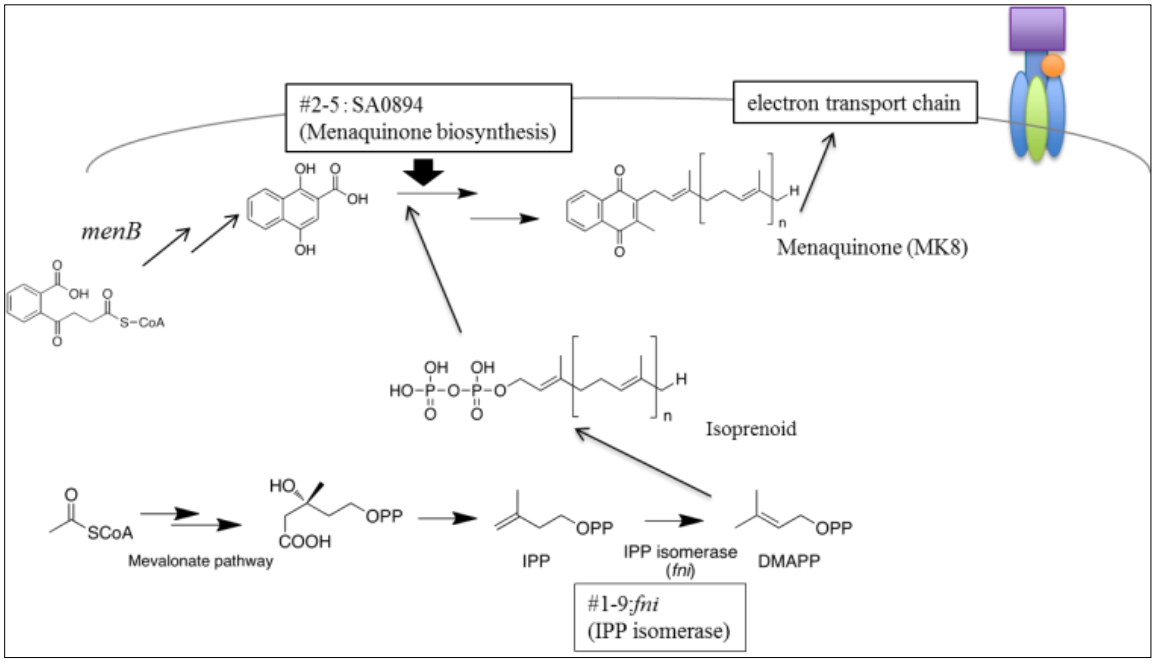



**Figure 13: Involvement of *fni* and *menA* genes in menaquinone biosynthetic pathway**

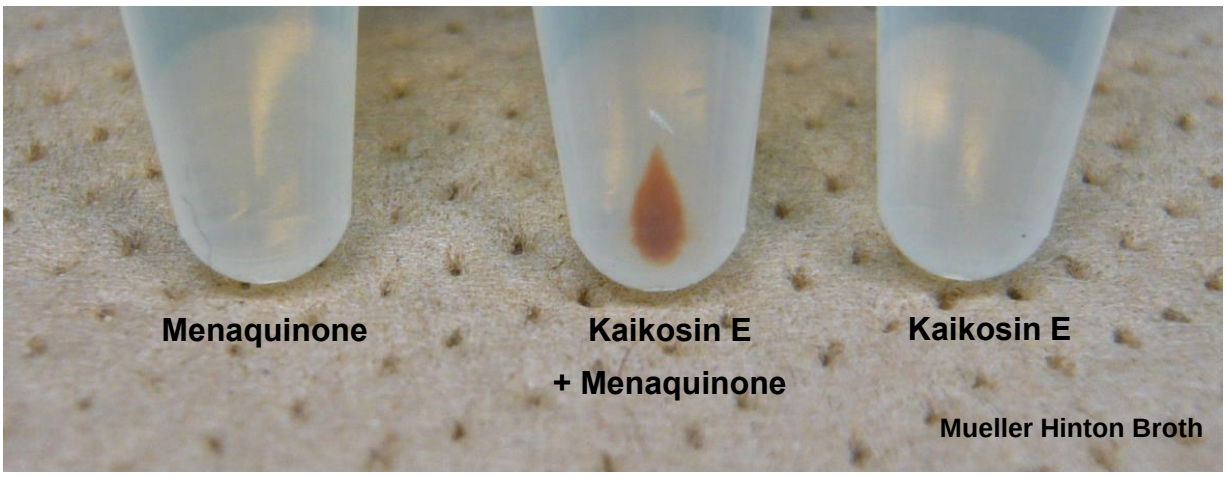



**Figure 14: Binding of kaikosin E with menaquinone.** 100 µg $ml^{-1}$ kaikosin E was mixed with 10 µg $ml^{-1}$ menaquinone (MK4) and centrifuged. Picture was immediately taken after centrifugation. The left tube is menaquinone alone, middle is kaikosin E mixed with menaquinone and right tube is kaikosin E alone.

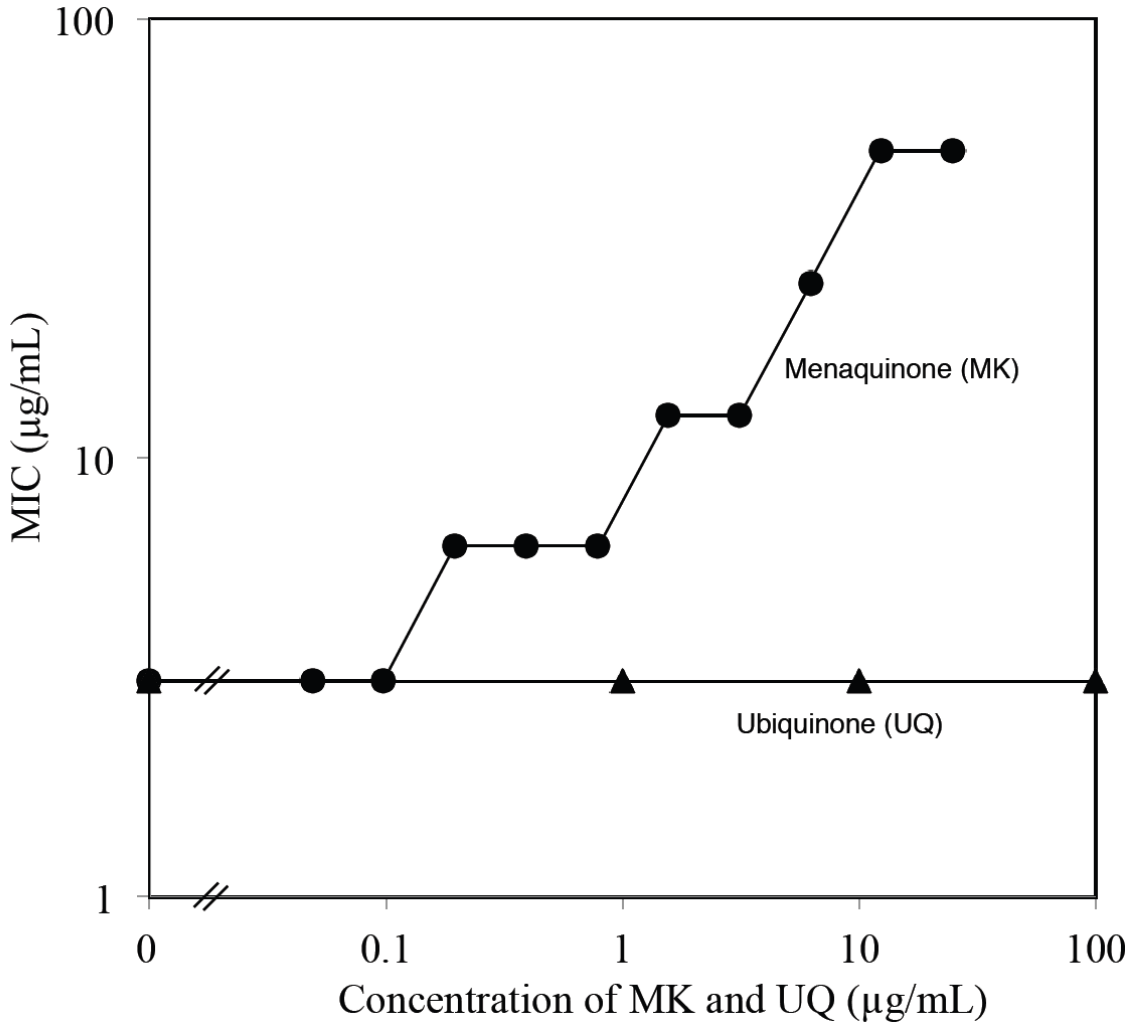



**Figure 15: Effect of addition of menaquinone on antimicrobial activity of kaikosin E.** Menaquinone (MK4) and ubiquinone were added externally to the assay system and MIC of kaikosin E against MSSA1 was determined by broth dilution assay.

**b. Mechanism of action of compound 363**

A total of 108 strains of EMS treated *S. aureus* were isolated from compound 363 containing agar plates. Among 108 resistant strains, two strains showed temperature sensitive phenotype: #74 and #108 that were obtained from 12.5 µg $ml^{-1}$ compound 363 containing agar plate **(Figure 16).** The MIC values of compound for these strains were found to be 4 and 8 fold higher respectively than that for the parent strain RN4220 **(Table 10).**

**Whole genome sequencing analysis**

The transformation efficiency of the TS mutants resistant to compound 363 was low, hence DNA library could not be transformed to find the gene(s) responsible for TS phenotype. Therefore, whole genome of the TS mutants was sequenced to identify the genes with mutations. Whole genome sequencing of the TS mutants #74 and 108 showed mutations in several genes including a common mutation in *sigA* (SA1390) gene **(Table 11).** The *sigA* gene had a common point mutation in the two mutants **(Table 11).** The *sigA* gene is an essential gene that encodes for RNA polymerase sigma factor (homologous to *rpoD* gene in *E. coli*) and is required for initiation of transcription of house-keeping genes. Mutation in this gene explains its RNA synthesis inhibitiory property. Further experiments were focused on this gene owing to the mutation in the same gene in two of the strains and its correlation with RNA synthesis inhibition. The slight inhibition of DNA synthesis can also be explained by mutation in this gene. RNA polymerase is also required for DNA synthesis, especially the lagging strand. Replication from 3' end to 5' end requires a short RNA primer which is initiated by RNA polymerase and later taken over by DNA polymerase. In this regard, DNA synthesis will also be slowly inhibited if RNA polymerase is inhibited by compound 363.

**Phage transduction**

Phage transduction was performed to determine if mutation in *sigA* gene was responsible for resistance to compound 363. Phage transduction is a bacteriophage mediated transfer of genetic material from one bacterium to another bacterium where homologous recombination takes place. In this case, phage harboring wild-type *sigA* gene and chloramphenicol resistant ($Cm^r$) gene as a marker was introduced to #74 and #108 TS mutants and selected in chloramphenicol containing agar plates. The scheme of phage transduction is shown in **Figure 17.** At first, a bacteriophage infects a donor bacterium, in this case *S. aureus*. It replicates inside the bacterial cell and acquires bacterial gene, here wild-type *sigA* gene and a $Cm^r$ gene as marker. The phage carrying the donor bacterium gene can now infect the recipient bacterium, in this case compound 363 resistant mutants. Inside the recipient bacterium, the phage inserts the gene from donor bacterium. Homologous recombination occurs and two combinations are possible, one is the wild-type (WT) *sigA* gene is exchanged with the mutant (MT) *sigA* gene along with the $Cm^r$, another is only $Cm^r$ marker is inserted in the mutant keeping its mutated *sigA* gene. When the transductants are selected in the presence of chloramphenicol, there exists two possibilities: one containing the wild-type *sigA* gene and $Cm^r$ marker and the other containing mutated *sigA* gene and $Cm^r$ marker. The percentage for each is 85% and 15% respectively which is a probability calculated from the distance between the marker and gene. Phage transduction resulted in 37 and 38 transductants for #74 and #108 respectively, out of which 29 and 33 transductants were susceptible to compound 363 owing to 78% and 87% respectively **(Table 12).** Theoretically, the expected value is 85% based on the distance of the gene from marker **(Figure 18).** The obtained values are similar to the expected value indicating that mutation

in *sigA* gene is responsible for resistance to compound 363. This result suggests that the RNA polymerase sigma factor might be involved in the antibacterial mechanism of compound 363.

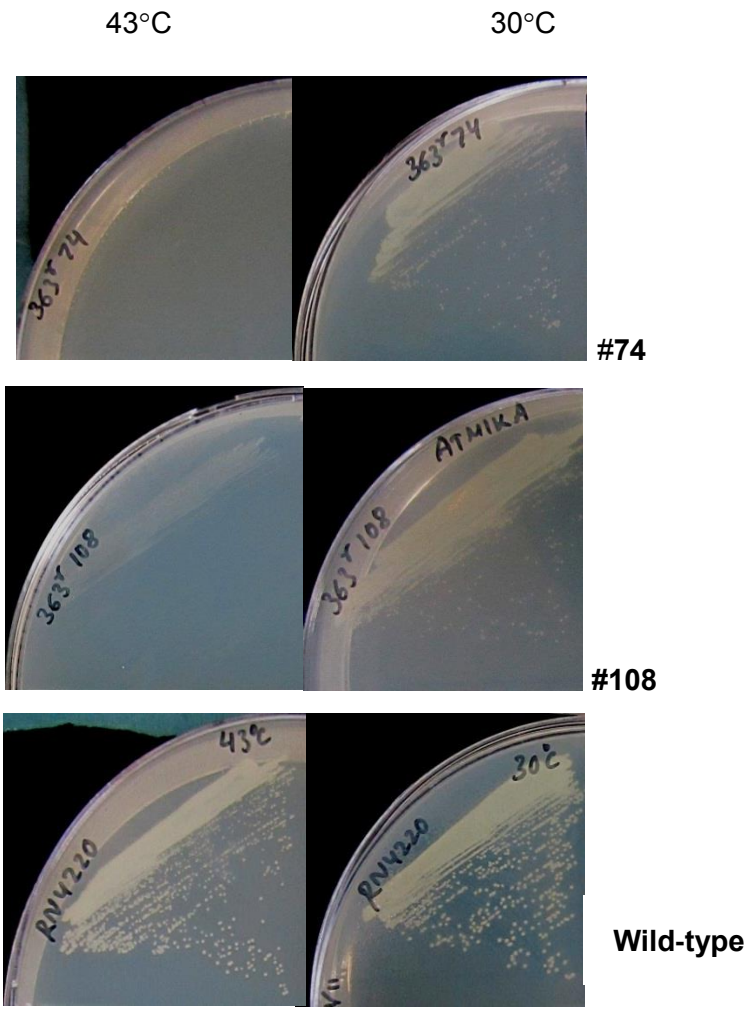



**Figure 16: Temperature sensitive compound 363 resistant strains.** The cells were grown in liquid LB medium overnight, diluted 100 fold with 0.9% NaCl, streaked on NaCl-depleted LB agar plates and incubated at 30°C and 43°C overnight.

| Strain | MIC (μg $ml^{-1}$) |
|---|---|
| RN4220 wild type | 12.5 |
| #74 | 25 |
| #108 | 50 |

**Table 10: MIC of 363 resistant TS mutants**

| #74 | #108 |
|---|---|
| *gatA* | *A1708* |
| *uppS* | *SA0940* |
| *capI* | *infB* |
| *SA1675* | *SA0544* |
| *sigA* (Asp201Asn) | *sigA* (Asp201Asn) |
| *sirC* | *uvrA* |
| *tgt* | |
| *dnaK* | |
| *SA1444* | |
| *adhE* | |
| *iunH* | |
| *SA0551* | |
| *thiE* | |
| *yycH* | |
| *metE* | |

*Underlined are the essential genes

**Table 11: Single gene mutations in 363 resistant TS mutants**

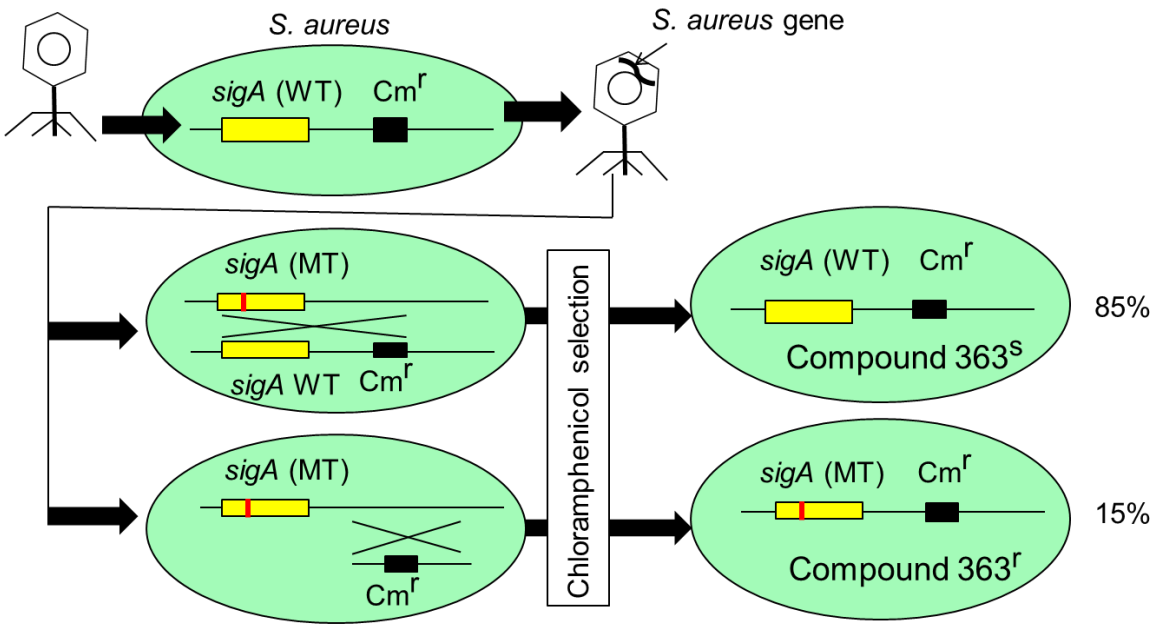



**Figure 17: Schematic representation of phage transduction of *sigA* gene**

| Transductants from strain | Susceptible to compound 363 |
|---|---|
| # 74 | 78% |
| # 108 | 87% |

**Table 12: Susceptibility of transductants to compound 363.**

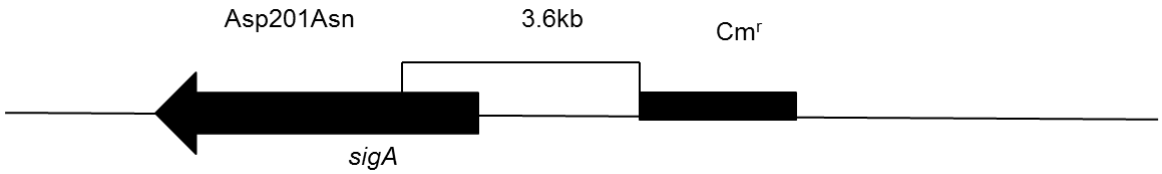



**Figure 18: Distance of marker from the mutation point in *sigA* gene**

**Conclusion:**

Here, cellular target of two novel antimicrobial agents identified using silkworm infection model was attempted to find out. Kaikosin E was found to have a novel target menaquinone that is selective to bacteria while compound 363 might target RNA polymerase sigma factor.

## PART 3

## Identification of novel antimicrobial agents against drug resistant bacteria

## Background

Compounds inhibiting the growth of drug resistant strains are the current need as the world is facing problem with the notorious drug resistant pathogens difficult to treat. Most of the therapeutic agents in clinical use today are either chemically synthesized or natural products. Screening for identification of lead compound and structure modification has been major strategy to enhance potency. Chemical synthesis (Smith et al., 2011), mutasynthesis (Anderle et al, 2008), and engineering the key enzymes (Panthee et al., 2011) required for biosynthesis have been used to modify the core structure of bioactive compounds. The success of each method relies on the appropriate research design and availability of screening system. Here chemical synthesis strategy was used to screen not only for enhanced antimicrobial activity but also reduced toxicity. Appropriate modifications in the chemical structures can lead to optimization of the compounds for desired effects such as bactericidal, less toxic and therapeutic effects.

## Results and discussion

### a. First library screening

A chemical library of 400 compounds synthesized for the purpose of total synthesis of natural products in the laboratory of Synthetic Natural Products Chemistry, Graduate School of Pharmaceutical Sciences, the University of Tokyo was screened for antibacterial activity. Among 400 compounds, 47 were found to possess antibacterial activity against *S. aureus*. Among them, the best hit FG050227 (**1**) was selected. Compounds with a structure similar to **1** (MIC: 3 μg $ml^{-1}$) were selected from the chemical library (**Figure 19**: **1-11**), from which two additional compounds FG050223 (**2**, MIC: 6 μg $ml^{-1}$), and FG050204 (**3**, MIC: 13 μg $ml^{-1}$)

showed antibacterial activities, whereas **4-11** had no antibacterial activity at a concentration of 500 μM (**Table 13**).

Most of the compounds, except **9**, had a deoxyribofuranosyl indole core. An additional indole ring is present next to deoxyribofuranosyl indole core in **1**, **3**, **7**, and **8** with a sulfonyl group attached to it only in **1.** The presence of iodine at the 2-position of the deoxyribofuranosyl indole core confers antibacterial activity (**2**) whereas an additional indole group in that position abolishes activity (**8**). Compound **7** similarly showed a loss of antimicrobial activity, whereas the addition of a sulfonyl group to the additional indole (**1**) restored activity. These findings suggest that the presence of an indole ring alone may reduce antibacterial activity while the addition of a strong electron-withdrawing group, like a sulfonyl group, can recover the activity. Further comparison between **3**, **7**, and **8** revealed that even in the presence of the additional indole ring, modification of the upper group attached to deoxyribofuranosyl indole core can lead to antibacterial activity.

**Antimicrobial spectrum of deoxyribofuranosyl indole compounds**

Compounds **1, 2**, and **3** were found to be effective against Gram-positive bacteria, including several clinical isolates of MRSA and VRE **(Table 13).** Compounds **1** and **2** exerted antifungal activity against pathogenic true fungi like *Candida albicans, Candida tropicalis*, and *Cryptococcus neoformans* with MIC values comparable to that against bacteria, whereas **3** showed much weaker antifungal activity compared to its antibacterial activity. The molecular targets of these compounds are expected to be different from that of vancomycin and methicillin as demonstrated by their similar potency against strains resistant and

sensitive to vancomycin and methicillin. Whereas compounds **1** and **2** showed similar potency against bacteria as well as fungi, **3** showed stronger activity against bacteria than fungi.

**Effect on macromolecule biosynthesis in *S. aureus***

Compounds **1** and **3** inhibited the incorporation of [$^{3}$H]N-acetylglucosamine **(Figure 20a),** [$^{3}$H]thymidine **(Figure 20b),** [$^{3}$H]uridine **(Figure 20c)** and [$^{35}$S]methionine **(Figure 20d**), suggesting that they inhibit the biosynthesis of peptidoglycan, DNA, RNA, and protein. Compound **2** inhibited the incorporation of [$^{3}$H]uridine **(Figure 20c)** and [$^{35}$S]methionine **(Figure 20d),** whereas its inhibitory effect on the incorporation of [$^{3}$H]N-acetylglucosamine **(Figure 20a)** and [$^{3}$H]thymidine **(Figure 20b)** was not significant. Compound **2** differed from compounds **1** and **3** in the inhibition pattern of macromolecule synthesis, suggesting that these compounds have different cellular targets.

**Bactericidal activity against *S. aureus***

Compounds **1** and **3** exerted potent bactericidal activity immediately after the addition of these compounds **(Figure 21).** The bactericidal activity of these two compounds was stronger than that of daptomycin. Compound **2** did not kill bacteria, indicating that action of this compound is bacteriostatic **(Figure 21).** Bacteriostatic agents, unlike bactericidal agents, inhibit bacterial growth and do not kill bacteria, demanding frequent introduction of the agent, and are thus not the appropriate choice for the treatment of serious, life threatening infections. In this study, structural modifications led to changes from bacteriostatic to bactericidal activity.

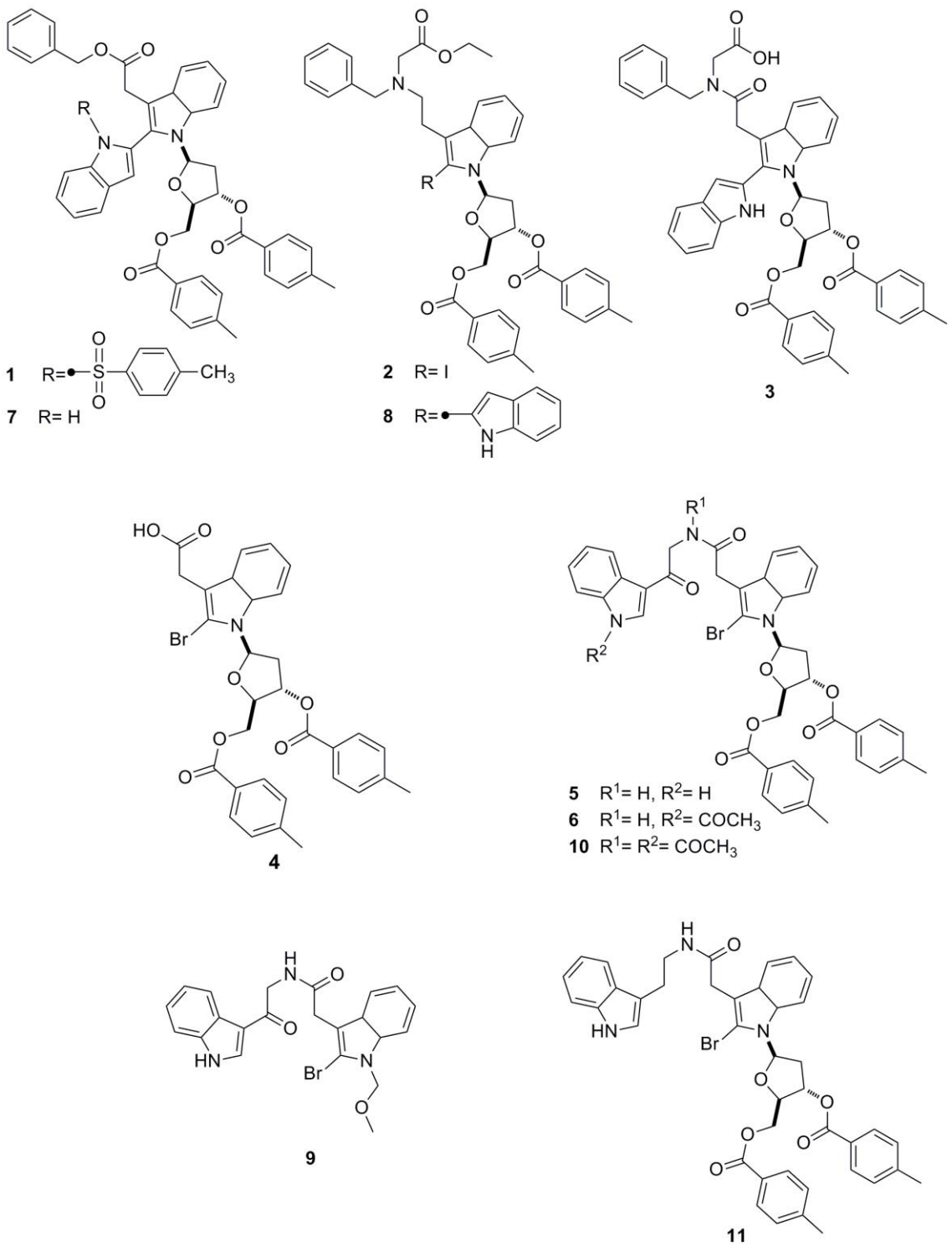
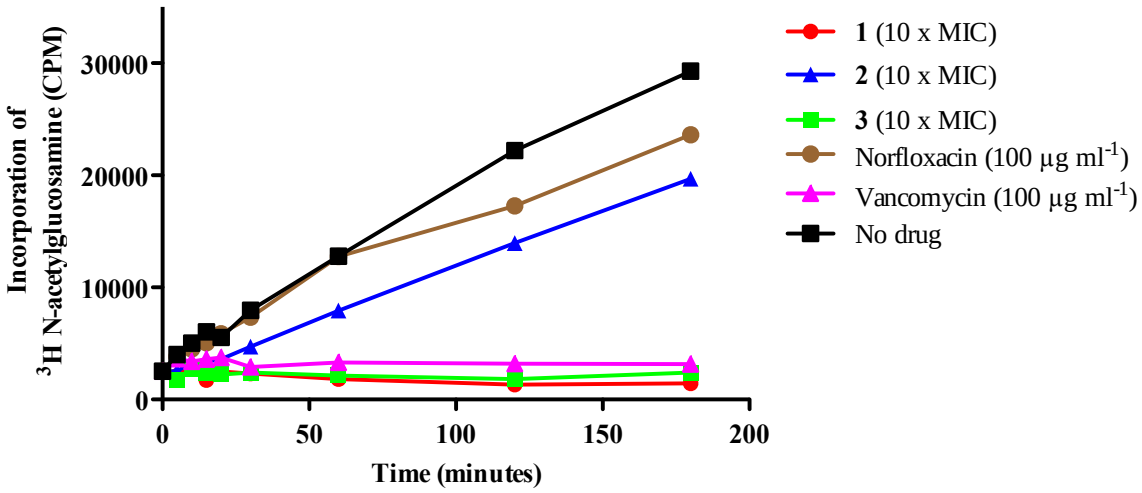
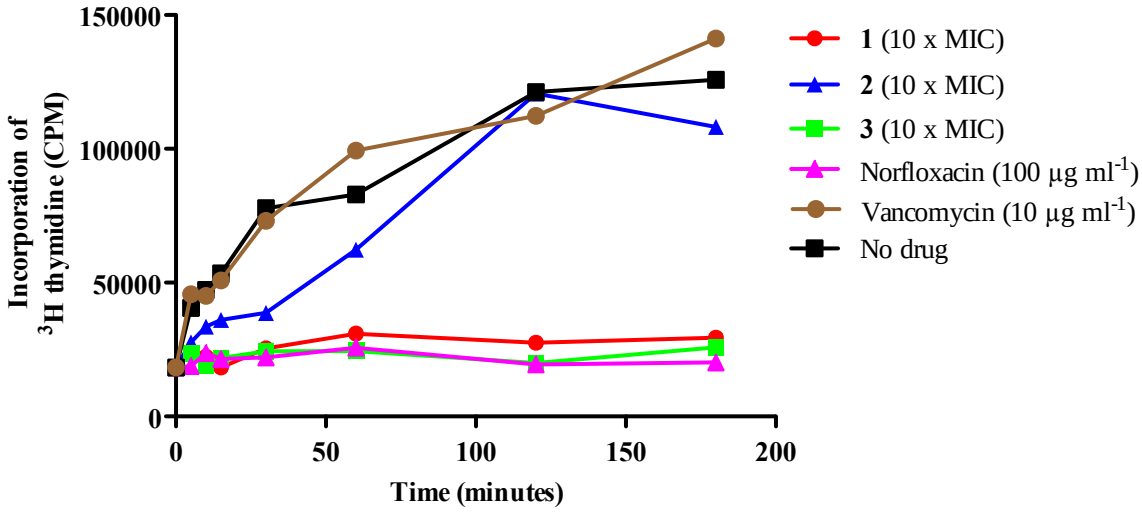



**Figure 19: Compounds used in this study**

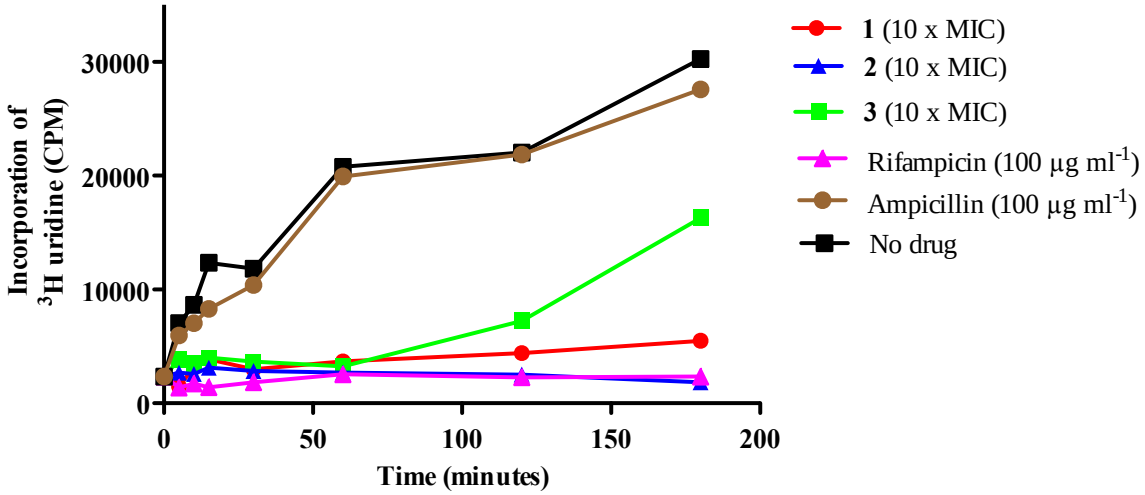
| Strain | MIC (μg ml$^{-1}$) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** |
| *S. aureus* MSSA1 | 3 | 6 | 13 | >300 | >380 | >400 | >360 | >400 | >230 | >420 | >370 |
| *S. aureus* RN4220 | 3 | 6 | 13 | - | - | - | - | - | - | - | - |
| *S. aureus* NCTC8325 | 3 | 6 | 13 | - | - | - | - | - | - | - | - |
| *S. aureus* Smith | 3 | 6 | 13 | - | - | - | - | - | - | - | - |
| Methicillin-resistant *S. aureus* MRSA3 | 3 | 6 | 13 | - | - | - | - | - | - | - | - |
| Methicillin-resistant *S. aureus* MRSA4 | 3 | 6 | 13 | - | - | - | - | - | - | - | - |
| Methicillin resistant *S. aureus* MRSA6 | 3 | 6 | 13 | - | - | - | - | - | - | - | - |
| Methicillin resistant *S. aureus* MRSA8 | 3 | 6 | 13 | - | - | - | - | - | - | - | - |
| Methicillin resistant *S. aureus* MRSA9 | 3 | 6 | 13 | - | - | - | - | - | - | - | - |
| Methicillin resistant *S. aureus* MRSA11 | 3 | 6 | 13 | - | - | - | - | - | - | - | - |
| Methicillin resistant *S. aureus* MRSA12 | 3 | 6 | 13 | - | - | - | - | - | - | - | - |
| *Enterococcus faecals* EF1 | 3 | 6 | 13 | - | - | - | - | - | - | - | - |
| Vancomycin resistant *Enterococcus faecalis* EF5 (VRE) | 3 | 6 | 13 | - | - | - | - | - | - | - | - |
| *Bacillus subtilis* JCM2499 | 3 | 3 | 6 | - | - | - | - | - | - | - | - |



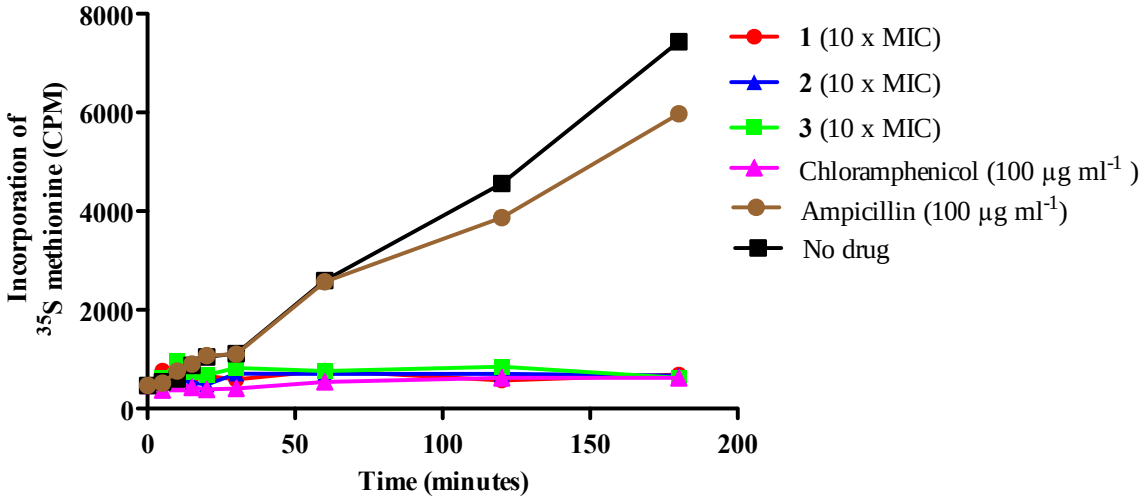

**a. Effect on Peptidoglycan synthesis**




**b. Effect on DNA synthesis**




**c. Effect on RNA synthesis**




**d. Effect on protein synthesis**

**Figure 20: Effect of compounds 1, 2 and 3 on macromolecule biosynthesis.** Radiolabeled precursors were added to expontentially growing *S. aureus* in presence or absence of antibiotics. Samples were collected at different time intervals as indicated and radioactivity of acid insoluble fractions were measured by liquid scintillation counter.

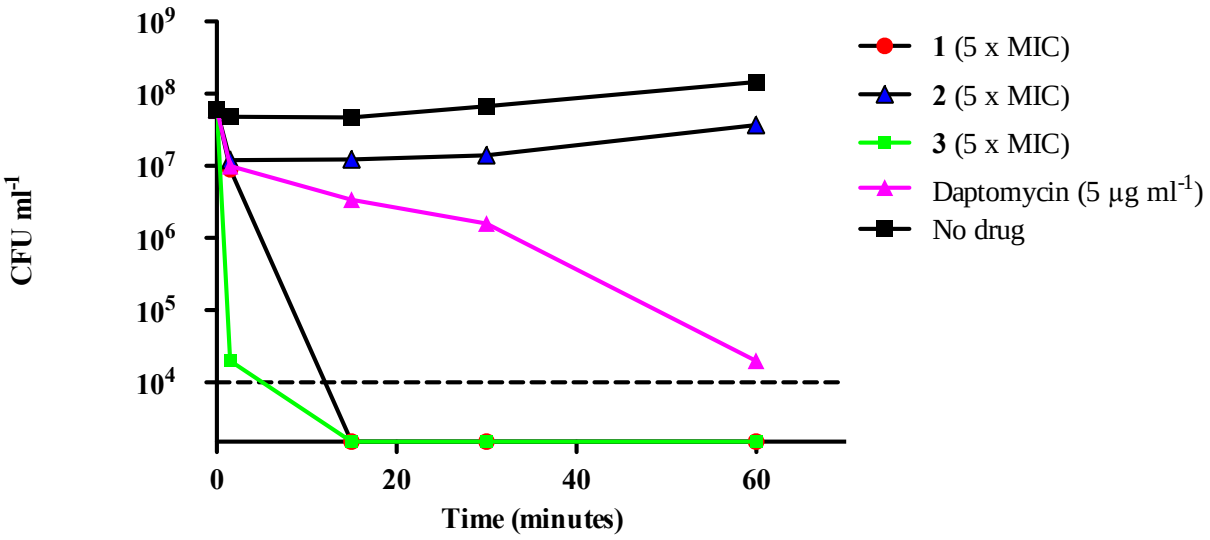



**Figure 21: Killing assay of compounds 1,2, and 3.** Exponentially growing *S. aureus* was treated with antibiotics. Culture aliquots were collected at different time as shown, diluted and spread on agar plates and incubated at 37°C for 24 hours. Cell viability was determined by counting the CFU $ml^{-1}$. Dotted line is the detection limit of this experiment.

**b. Second library screening**

A total of 103873 compounds from the open innovation center for drug discovery, the University of Tokyo were screened for antimicrobial activity against *S. aureus.* 0002-04-KK (**12**) had antibacterial activity against *S. aureus,* MSSA1 (25 μg $ml^{-1}$) and it was toxic to silkworm model with $LD_{50}$ value of 100 μg g•$larva^{-1}$.

**Structure activity relationship of 12**

With an aim to enhance the antibacterial activity, and reduce the toxicity of **12**, structure activity relationship (SAR) of this compound was analyzed. Toxicity was tested using silkworms as model animal. Imino-thaidiazolo-pyrimidinone **(Figure 22)** was taken as the mother structure while structures at $R^1$ and $R^2$ positions were modified to obtain nine different compounds. **Table 14** shows the SAR analysis. It was found that 0026-59-KK (**13**) had strongest antibacterial activity against *S. aureus* and the least toxicity to silkworms. Hence, this compound was selected for further experiments.

**Antimicrobial spectrum of 12 and 13 and toxicity to silkworm model**

Both the compounds showed antimicrobial activity against Gram-positive bacteria including resistant strains such as MRSA and VRE, **13** had potent activity compared to **12 (Table 14).** **12** also had weak activity against some fungi except *Aspergillus niger* while **13** did not exert antifungal activity. SAR analysis showed that **13** harbouring benzene ring and nitrofuran moiety in $R^1$ and $R^2$ positions respectively, was the most effective against *S. aureus* with least toxicity to silkworm. Presence of hydrogen (0005-08-KK) and furan ring (0023-37-KK) led to decreased antibacterial activity **(Table 15).** In other cases, the antibacterial activity

was not affected while toxicity was higher in trifluoro-methane containing groups. A closer comparison of the structures revealed trifluoro-methane moiety to be responsible for toxicity except in 0019-25-KK and 0027-56-KK **(Table 14).** In these compounds, the combination of $R^1$ and $R^2$ moieties might be responsible to counter act each other's effects. The toxicity of trifluoro-methane moiety was further verified by the antifungal activity possessed by **12.**

**Mechanism of action of 12 and 13**

Both **12** and **13** inhibited the incorporation of radiolabeled precursors [$^3$H]thymidine, [$^3$H]N-acetyl glucosamine, [$^3$H]uridine, and [$^{35}$S]methionine within 10 minutes in exponentially growing *S. aureus* suggesting the inhibition of macromolecules DNA, peptidoglycan, RNA, and protein respectively **(Figure 23).**

Interestingly, **12** exerted bacteriostatic while **13** exerted bactericidal activities (**Figure 24**). The bactericidal activity of **13** was rapid and it killed bacteria within 30 minutes. The killing activity was comparable to that of daptomycin. In order to get more insight and trying to find out which moiety was responsible for the killing action, other two compounds; 0006-09-KK (**14**) and 0027-56-KK (**15**) were selected and killing assay was performed. It was found that **15** exhibited bactericidal activity and **14** showed bacteriostatic activity. The fact that **14** was bacteriostatic while **15** was bactericidal revealed that nitrofuran moiety was responsible for the killing action.

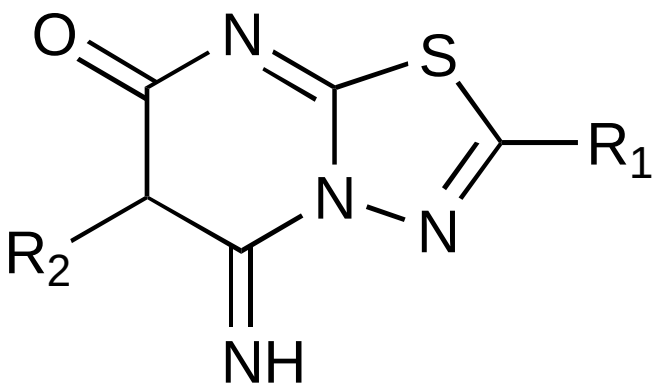
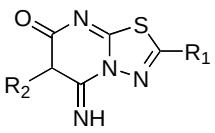
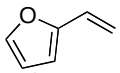
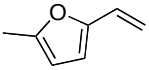
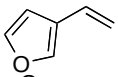
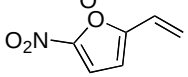
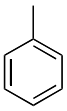
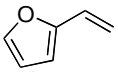
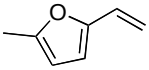
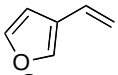
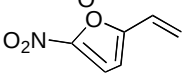



**Figure 22: Mother structure of imino-thaidiazolo-pyrimidinone**



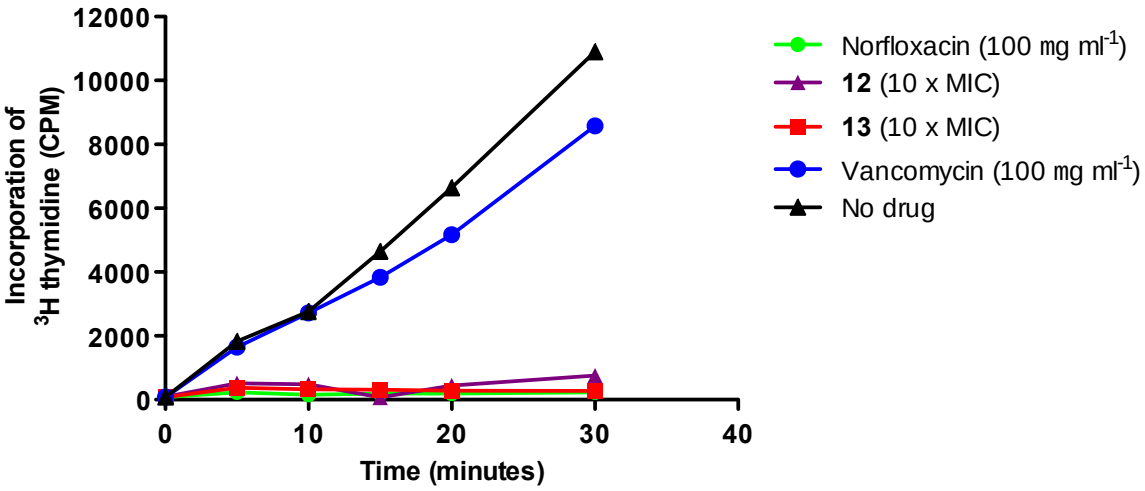
| $R_1$ | $R_2$ | Compound name | MIC (µg $ml^{-1}$) | $LD_{50}$ (µg $g.larva^{-1}$) | Mode of action |
|---|---|---|---|---|---|
| $F_3$ | H | 0001-03-KK | 25 | 62 | |
| |  | 0002-04-KK (**12**) | 25 | 120 | Bacteriostatic |
| |  | 0017-22-KK | 12.5 | 120 | |
| |  | 0021-27-KK | 25 | 77 | |
| |  | 0027-56-KK (**15**) | 25 | 200 | Bactericidal |
|  | H | 0005-08-KK | > 400 | N.D. | |
| |  | 0006-09-KK (**14**) | 25 | >150 | Bacteriostatic |
| |  | 0019-25-KK | 12.5 | 27 | |
| |  | 0023-37-KK | 400 | > 270 | |
| |  | 0026-59-KK (**13**) | 6.3 | > 270 | Bactericidal |

N.D.: Not determined

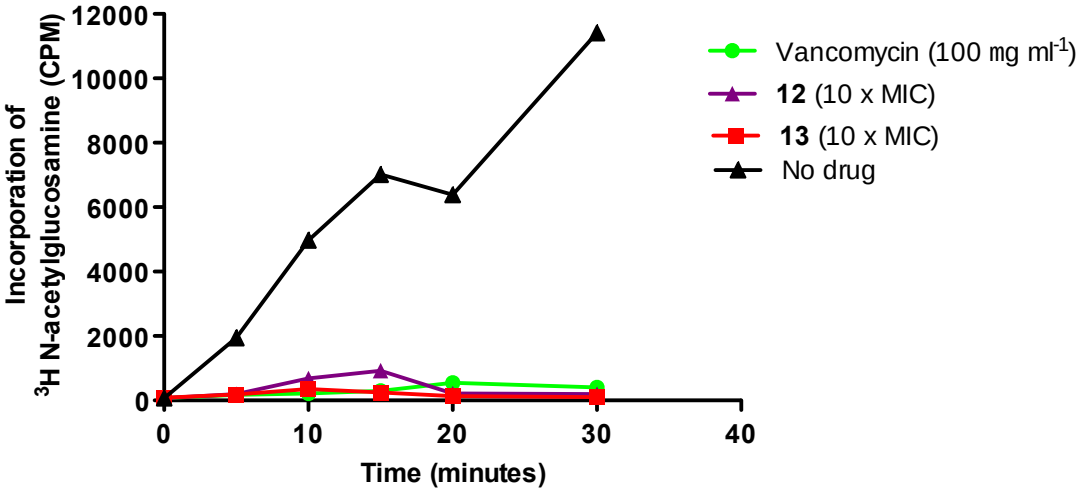
**Table 14: Structure activity relationship of the compounds**

| Strain | MIC (μg ml$^{-1}$) | |
|---|---|---|
| | **12** | **13** |
| *S. aureus* MSSA1 | 50 | 6.3 |
| *S. aureus* RN4220 | 50 | 3.1 |
| *S. aureus* NCTC8325 | 50 | 3.1 |
| *S. aureus* Smith | 50 | 6.3 |
| Methicillin-resistant *S. aureus* MRSA3 | 50 | 6.3 |
| Methicillin-resistant *S. aureus* MRSA4 | 50 | 6.3 |
| Methicillin-resistant *S. aureus* MRSA6 | 50 | 6.3 |
| Methicillin-resistant *S. aureus* MRSA8 | 50 | 6.3 |
| Methicillin-resistant *S. aureus* MRSA9 | 25 | 6.3 |
| Methicillin-resistant *S. aureus* MRSA11 | 50 | 3.1 |
| Methicillin-resistant *S. aureus* MRSA12 | 50 | 6.3 |
| *Enterococcus faecalis* EF1 | 100 | 25 |
| Vancomycin-resistant *Enterococcus faecalis* EF5 (VRE) | 100 | 12.5 |
| *Bacillus subtilis* JCM2499 | 100 | 6.3 |
| *Bacillus cereus* JCM20037 | 100 | 3.1 |
| *Streptococcus sangunius* JCM5708 | >100 | 25 |
| *S. pyogeneus* SS1-9 | >100 | 25 |
| *S, agalactine* JCM5671 | >100 | 12.5 |
| *S. pneumoniae* JCSC6523 | 50 | 1.5 |
| *Serratia marcescens* 98-130-37 | >100 | >100 |
| *Escherichia coli* W3110 | >100 | >100 |
| *Pseudomonas aeruginosa* PAO1 | >100 | >100 |
| *Candida albicans* ATCC10231 | 100 | >100 |
| *C. tropicalis* pK233 | 100 | >100 |
| *Cryptococcus neoformans* H99 | 100 | >100 |
| *Aspergillus niger* | >100 | >100 |

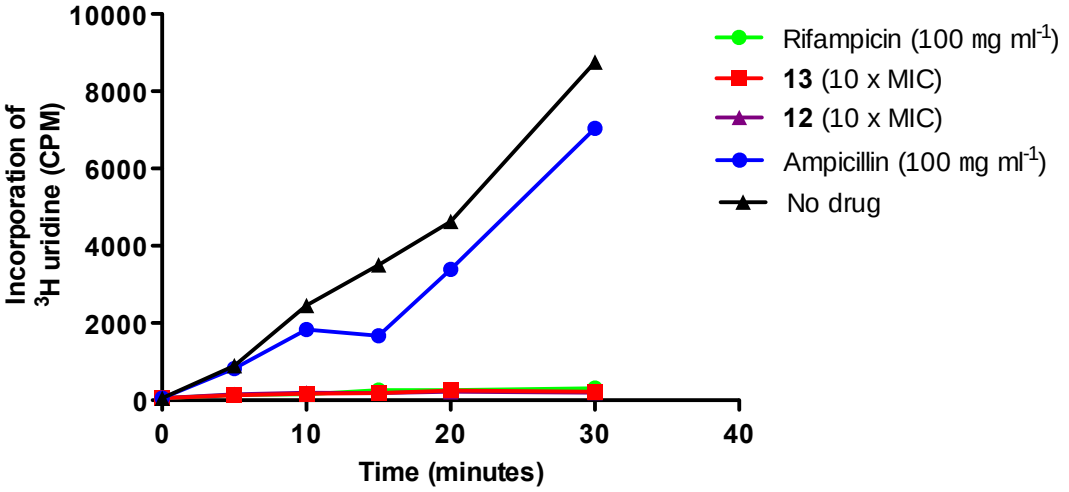
**Table 15: Minimum inhibitory concentration (MIC) of the compounds against microoganisms**



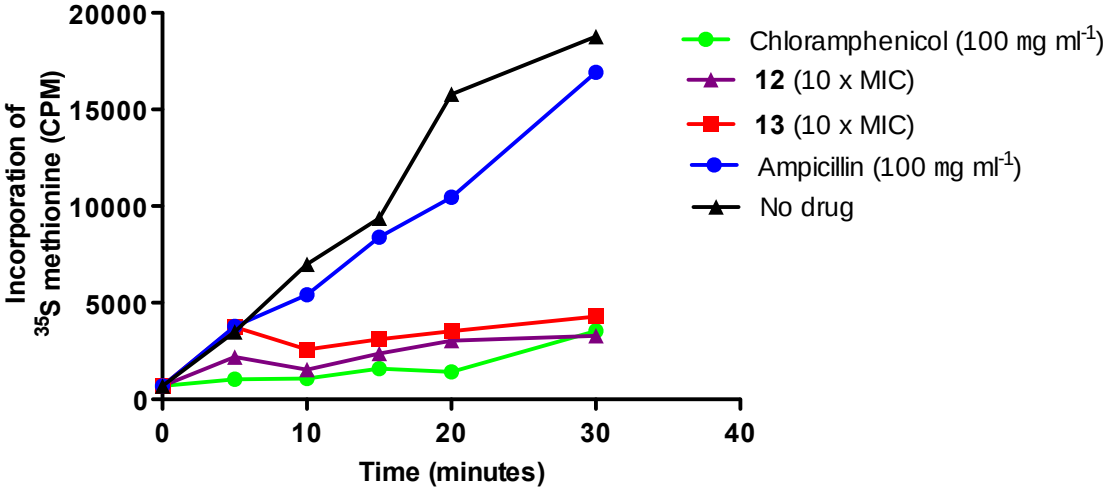

**a. Effect on DNA synthesis**




**b. Effect on peptidoglycan synthesis**




**c. Effect on RNA synthesis**




**d. Effect on protein synthesis**

**Figure 23: Effect of compounds 12 and 13 on macromolecule biosynthesis.** Radiolabeled precursors were added to expontentially growing *S. aureus* in presence or absence of antibiotics. Samples were collected at different time intervals as indicated and radioactivity of acid insoluble fractions were measured by liquid scintillation counter.

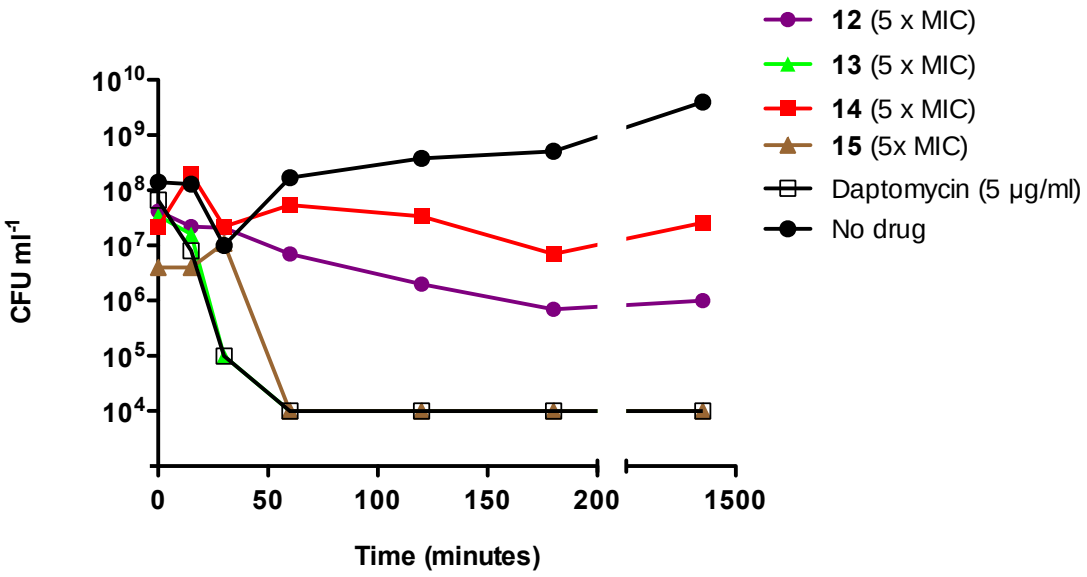



**Figure 24: Killing assay for compounds 12, 13, 14, and 15. .** Exponentially growing *S. aureus* was treated with antibiotics. Culture aliquots were collected at different time as shown, diluted and spread on agar plates and incubated at 37°C for 24 hours. Cell viability was determined by counting the CFU $ml^{-1}$.

**Conclusion**

In conclusion, these compounds are potential lead compounds for the development of antimicrobial agents. Further structural modifications can be made as required with suitable combinations to expect antimicrobial properties and bactericidal activities. This kind of SAR study is helpful in drug discovery and therapeutics.

# MATERIALS AND METHODS

**Microbial strains and culture conditions**

The bacterial and fungal strains used in this study are summarized in **Table I.** Bacterial cultures were prepared in either Luria Bertani (LB) medium (tryptone 10 g $l^{-1}$, yeast extract 5 g $L^{-1}$, NaCl 10 g $L^{-1}$) or Mueller-Hinton Broth (MHB) (DIFCO). For antimicrobial susceptibility tests, cation-adjusted MHB was used. Fungal suspensions were prepared in Roswell Park Memorial Institute (RPMI) medium (Sigma Aldrich, St. Louis, MO, USA).

**Chemicals and reagents**

Vancomycin, daptomycin, and norfloxacin were purchased from Wako Pure Chemicals (Japan), Sequoia Research Products (UK), and Sigma Aldrich, respectively. Rifampicin and chloramphenicol were obtained from Nacalai Tesque (Japan). Radiolabelled [$^{3}$H]N-acetylglucosamine was obtained from GE Healthcare (UK), [methyl-$^{3}$H]thymidine and [$^{3}$H]uridine were obtained from Moravek Biochemical (Brea, CA, USA), and [$^{35}$S]methionine was obtained from the Institute of Isotopes (Hungary). $DiSC_3(5)$ was purchased from Molecular Probes, Eugene, Oreg. Ethylmethansesulfonate (EMS) was obtained from Sigma Aldrich, St. Louis, MO, USA. All the chemicals used were of analytical grade.

**Determination of antimicrobial activity**

Antimicrobial activity was measured as minimum inhibitory concentration (MIC) values. MIC values of samples against different microorganisms were determined by broth dilution assay as described in CLSI (2009) and CLSI (1997) for antibacterial and antifungal assays respectively. Briefly, bacterial MIC was determined in cation adjusted Muller Hinton Broth (CAMHB: Mueller Hinton 10.5 g $L^{-1}$ containing 10mg $Ca^{2+}$ $ml^{-1}$ and 10mg $Mg^{2+}$ $ml^{-1}$). Bacteria

were diuted in CAMHB to get a final CFU of $5x10^5$ CFU $ml^{-1}$ in each well of 96-well plate. Drugs were added and serially diluted. The plates were incubated for 16-20 hours at 35±2°C before reading the end point. For the growth of *Listeria maltophilia, Streptococcus pneumoniae, Streptococcus sanguinis and Streptococcus agalactiae*, 10% horse serum was used. For antifungal assay, fungal innocula were prepared by picking five colonies of > 1mm diameter from 24-hour old culture of Candida species and 48- hour old culture of *Cryptococcus neoformans* grown on sabouraud agar (tryptone 10g, glucose 40g, agar 15 $gl^{-1}$). The colonies were suspended in 0.9% NaCl, vortexed and adjusted to $5.0x10^2$ CFU $ml^{-1}$. After drug addition and serial dilution, the plates were incubated at 35±2°C for 46-50 hours for others and 70-74 hours for *C. neoformans* before reading MIC.

**Preparation of silkworm infection model**

Silkworm eggs (Hu·Yo x Tukuba·Ne) were hatched and fed artificial diet with antibiotics (Silkmate 2S) until they developed to fifth-instar larvae. Then, they were fed an antibiotic-free artificial food (Silkmate) for one day. *S. aureus* grown in LB (Luria Bertani: tryptone 10g, yeast extract 5g, NaCl 10g, agar 15 g $L^{-1}$) medium was diluted with 0.9% NaCl to $3x10^7$ CFU and was injected into the hemolymph of each silkworm through the dorsal surface.

**Determination of therapeutic activity in silkworm**

The samples showing *in-vitro* antimicrobial activities were dissolved in suitable solvent (MilliQ or 0.9% NaCl or 20% DMSO) and 50 µl of it was injected into the silkworm hemolymph immediately after the injection of *S. aureus*. After injection, silkworms were placed in a safety cabinet without food. Number of survival was counted two days post

injection.

**Assessment of therapeutic activity in mouse infection model**

One ml culture of Smith strain of *Staphylococcus aureus* ATCC 13072 grown on Tryptic Soy Broth (Tryptic Soy Broth powdered medium: 30 g $l^{-1}$) overnight was centrifuged at 14000 rpm at 4°C for five minutes. The pellet was dissolved in one ml 7% mucin containing $FeNH_4$. Citrate. Mice, (ICR, four weeks old female; w=20 g) were injected intraperitoneally with 200µl of this suspension [CFU=$6.2x10^6$ ($20xLD_{50}$)]. The mice were fed food and water freely. After 2 hours of infection, they were injected with vancomycin, (50 µg, 25 µg, 12.5 µg and 6.25 µg per mouse) and kaikosin E (50 µg, 25 µg, 12.5 µg and 6.25 µg per mouse) or compound 363 (100 mg $kg^{-1}$) by subcutaneous route and the number of survival was counted and $ED_{50}$ values were determined.

**Thermo-stability of kaikosin E**

Kaikosin E 200 µg $ml^{-1}$ was autoclaved at 121°C for 20 minutes; MIC against MSSA1 was compared with untreated kaikosin E.

**Incorporation of [$^3$H]N-acetylglucosamine into cell wall peptidoglycans**

Measurement of incorporated [$^3$H]N-acetylglucosamine was performed as explained previously according to Paudel et al. Briefly, *S. aureus* NCTC8325 was cultivated at 37°C overnight in CGPY broth ($Na_2HPO_4$, 6 g; NaCl, 3 g; $MgCl_2.6H_2O$, 0.1 g; $NH_4Cl$, 2 g; $Na_2SO_4$, 0.15 g; $KH_2PO_4$, 3 g; Bactopeptone, 10 g; yeast extract, 0.1 g; and glucose, 5 g [per liter], pH 7.0). The culture was diluted 100-fold with the same medium and further cultivated until

$OD_{660}$ of 0.2 was reached. The culture was centrifuged at 8,000 g for 10 minutes and the pellet was suspended in modified cell wall synthesis medium ($KH_2PO_4$ [6 g], $K_2HPO_4$ [6 g], $NH_4Cl$ [2 g], $MgSO_4$. $7H_2O$ [5 mg], $FeSO_4$ [5 mg], glucose [100 mg], uracil [40 mg], L-alanine [50 mg], L-glutamic acid [120 mg], L-lysine [50 mg], chloramphenicol [100 mg] [per liter]) to obtain $OD_{660}$= 0.1. To 1 ml of the cell suspension, each of the samples under study (10xMIC) or vancomycin (100 µg $ml^{-1}$) or norfloxacin (100 µg $ml^{-1}$) were added in the presence of 35 µci [$^3$H]N-acetyl glucosamine and incubated at 37°C with shaking. Samples were collected at indicated time and equal volume of 10% trichloroacetic acid (TCA) was added. The mixture was incubated at 90°C for 15 minutes, placed on ice for 30 minutes, and filtered with a membrane filter (0.45 µm HA, Millipore, Ireland) followed by 5% TCA washing. The radioactivity was counted with a liquid scintillation counter (LS6000SE, Beckman Coulter, USA).

**Incorporation of radiolabeled thymidine, uridine, and methionine**

Incorporation of thymidine, uridine, and methionine was measured as explained previously (Paudel et al., 2012). Briefly, *S. aureus* RN4220 was grown overnight in LB medium overnight at 37°C. The culture was diluted 200-fold in the same medium and incubated at 37°C until an $OD_{600}$ of 0.3 was reached. Either 7 µci [$^3$H]uridine, 70 µci [$^3$H]thymidine, or 20 µci [$^{35}$S]methionine was added to the culture and further incubated at 37 °C for 10 minutes. Rifampicin (100 µg $ml^{-1}$), norfloxacin (100 µg $ml^{-1}$) and chloramphenicol (100 µg $ml^{-1}$) were used as inhibitors of RNA, DNA, and protein synthesis respectively, and vancomycin (10 µg $ml^{-1}$) or ampicillin (100 µg $ml^{-1}$) was used as negative control. Samples (10xMIC) were used for all the assays. Aliquots were collected at the indicated time, diluted 2 times by 5% TCA,

and the acid insoluble fraction was obtained by filtration through glass fiber filters (Whatman, GE Healthcare, UK). Radioactivity retained on the filters was measured by a liquid scintillation counter.

**Killing assay**

The killing assay was performed as per NCCLS guidelines. Briefly, overnight culture of *S. aureus* MSSA1 at 37°C in MHB was diluted 1000 times with the same medium and cultured for 2 hours at 37°C. For daptomycin, MHB was supplemented with 50 mg $l^{-1}$ $Ca^{2+}$. Kaikosin E (as indicated) or compound 363 (5xMIC) or daptomycin (5 µg $ml^{-1}$) were added to 1 ml of the culture and incubated at 37°C for 24 hour. Culture aliquots were collected at indicated time, diluted, and spread on LB agar plates and incubated at 37°C for 24 hours. Cell viability was determined by counting the CFU $ml^{-1}$. The lower limit of detection was $10^4$ CFU $ml^{-1}$.

**Bacteriolytic assay**

Overnight culture of MSSA1 at 37°C in Cation Adjusted Muller Hinton Broth was diluted 10 times with the same medium. Kaikosin E (25 µg $ml^{-1}$), vancomycin (10 µg $ml^{-1}$), daptomycin (10 µg $ml^{-1}$) were added to each 2 ml of this suspension and cultured at 37°C with shaking. Culture aliquots were collected at 10, 20, 30, 60, 120, and 180 minutes and $OD_{600}$ was measured.

**Dissipation of membrane potential**

Membrane potential was measured by using a fluorescent assay based on the method of Wu and Hancock (1999). *S. aureus* cultures grown to the early exponential phase (optical

density at 600 nm [$OD_{600}$], 0.2 to 0.3) in MHB were used. Aliquots (2 ml) were transferred to a polystyrene fluorimeter cuvette containing a stir bar and placed in the heated (37°C) sample chamber of a spectrofluorometer. The cells were excited at 622 nm, and the fluorescence emission was collected at 670 nm. Background data were collected for 30 s before the addition of $DiSC_3(5)$ (3,3'-dipropylthiadicarbocyanine iodide) to a final concentration of 100 nM. Data were collected for an additional 2 minutes after the addition of the fluorescent dye. After 2 minutes, it was treated with nisin (25 µg $ml^{-1}$), chloramphenicol (50 µg $ml^{-1}$), Kaikosin E (as indicated) or no drug at 37°C. The fluorescence emission was collected for additional 8 minutes.

**Screening of natural product**

Soil samples were collected from different parts of Japan and bacteria were isolated form them. The isolated bacteria were cultured in suitable broth for 5 days at 30˚C, and treated with 50% acetone. The crude samples thus obtained were tested for *in- vitro* antimicrobial activities against *S. aureus* by the broth dilution method and samples that inhibited visible growth of *S. aureus* were selected. Selected samples were injected into the hemolymph of silkworms infected with *S. aureus* and the survival rate of silkworms was determined after 2 days of incubation at 27°C.

**Isolation of drug resistant strains**

Wild type *S. aureus* RN4220 strain was treated with 0.2% mutagen ethylmethanesulfonate (EMS) and cultured in Tryptic soy broth (TSB) overnight at 30°C and 150 rpm. The culture was diluted 100 fold with the same medium and grown for further 9 hours at 30°C. The

resulting outgrowth was then diluted 10 fold with 0.9% NaCl and 100 µl of it was spread on TSB agar plates containing different concentrations of kaikosin E (2.5 µg $ml^{-1}$, 5 µg $ml^{-1}$, 10 µg $ml^{-1}$ , 20 µg $ml^{-1}$ and 40 µg $ml^{-1}$) or compound 363 (1.6 µg $ml^{-1}$, 3.2 µg $ml^{-1}$, 6.3 µg $ml^{-1}$, 12.5 µg $ml^{-1}$, 25 µg $ml^{-1}$, and 50 µg $ml^{-1}$) and incubated at 30°C overnight.

**Screening for temperature sensitive strains among the resistant strains**

TS mutants were isolated as previously described (Inoue et al., 2001). Briefly, kaikosin E or compound 363 resistant strains were grown on LB medium at 30°C overnight and 150 rpm. The resulting cultures were diluted 100 fold with 0.9% NaCl and streaked on NaCl- depleted LB agar plates. Each strain was streaked on two plates and grown at two different temperatures: 43°C and 30°C overnight. Temperature sensitive strain would grow at 30°C while would not grow at 43°C in contrast to the wild-type which would grow at both the temperatures.

**Preparation of competent cells**

TS mutants were grown on LB medium at 30°C 150 rpm overnight. Two ml of the resulting culture was diluted 100 fold and grown at 30°C and 150 rpm until $OD_{600}$ reached 0.3. The culture was then kept on ice for 5 minutes and centrifuged at 8krpm for 10 minutes at 4°C. The resulting precipitate was suspended in 50 ml 500 µM sucrose on ice and centrifuged at 8krpm for 10 minutes at 4°C. The precipitate thus obtained was suspended in 500 µl of 500 µM sucrose and cooled on liquid Nitrogen and stored at -80°C immediately until used.

**Genetic analysis for identification of gene responsible for TS phenotype**

Gene responsible for TS phenotype was identified by transformation of DNA library of *S. aureus* to the TS mutants and sequencing. Vector pSR515 was used for all the cases.

**Transformation of DNA library**

*S. aureus* genome DNA library was transformed to the competent cells of the TS mutants by electroporation in a 0.2- cm cuvette at 2.5 Kilovolts, 25 μF and 100 Ω. B2 broth was immediately added to the cuvette and incubated at 30°C for 1 hour and 100 μl aliquots were spread on two NaCl–depleted LB agar plates containing 12.5 μg $ml^{-1}$ chloramphenicol and incubated at two different temperatures; 43°C and 30°C.

**Purification of plasmid DNA from *S. aureus***

The colonies that could grow at both the temperatures complementing the TS phenotype, i.e., colonies that were converted to temperature resistant (Tr) phenotype were isolated and their plasmid DNA was purified. For the plasmid DNA purification from *S. aureus*, overnight culture of the cells was harvested by centrifugation, treated with 0.5 μg $μl^{-1}$ lysostaphin, and incubated at 37°C for 30 minutes. Phenol/chloroform extraction was performed followed by ethanol precipitation.

**Extraction of genomic DNA**

Genomic DNA was extracted from the overnight culture of bacteria using QIAamp DNA blood mini kit (250) (Qiagen).

**Purification of plasmid from *E. coli***

Plasmids from *E. coli* were purified by phenol/chloroform extraction after treatment with 0.1 μg μl $^{-1}$ RNase followed by ethanol precipitation.

**Construction of plasmid and complementation analysis**

Wild type *menA* and *fni* genes harboring plasmids were constructed. The wild type *menA* gene was amplified from genomic DNA of RN4220 using primers 5'-GATACGTCAAGCACGTCCAG -3' and 5'-GTAGCCATCCATTTCACTTC-3' and *fni* using primers 5'-CATTTTGAAGACCGCCATGT-3' and 5'-TTGGCGATGTCGCATTAGGT-3' and inserted at the *Sma*I site of the vector PSR515, then cloned in *E. coli* Jm109. Plasmid DNA was extracted from *E. coli* and transformed into the corresponding TS mutants, then grown at 30°C and 43°C.

**DNA sequencing**

*menA* and *fni* genes were amplified using the same primers used for plasmid construction from RN4220 genomic DNA. For *sigA* gene, 5'-AAATAAGCATGATCTGAGCC-3' and 5'-AATTAAGGGAAGCTACAAGG-3' were used. The resulting PCR products were purified with QIAquick gel extraction kit (Qiagen) and used as template for sequence reaction. For sequencing, following primers were used in addition to the primers used for amplification: *menA*: 5'-GAGCCCAGAGCTTGTGCTAC-3' and 5'-GTAGCACAAGCTCTGGGCTC-3', *fni*: 5'-TTCAACTGCTTCCAAAGCCT-3' , 5'-GGGATCCAGTGATTTTCAAAGAGAACAGAG-3', and 5'-GGGATCCTCCTCGATGTATATTCAAGCTACG-3', and *sigA*: 5'-GTAGGTCGTGGTATGTTATT-3', 5'-AAGCAGCATGATCTGAAGG-3', 5'-

CTGCTGGTAAATCCATTTCT-3', and 5'-CAAGAAGGTAATATGGGTC-3'. Sequencing reactions were performed with PRISM BigDye terminator kit and analyzed by DNA sequencer (ABI 373A, Applied Biosystems).

**Menaquinone binding assay**

Menaquinone (10μg $ml^{-1}$) and kaikosin E (100μg $ml^{-1}$) were mixed in Mueller Hinton Broth for several seconds and centrifuged at 15 Krpm for 5 minutes at room temperature. Menaquinone (10 μg $ml^{-1}$) and kaikosin E (100 μg $ml^{-1}$) alone were used as control.

**Antibacterial activity of kaikosin E in presence of menaquinone**

MIC of kaikosin E against MSSA1 was determined by broth dilution assay in presence of menaquinone (0.05 μg $ml^{-1}$ - 25 μg $ml^{-1}$) or ubiquinone (0.05 μg $ml^{-1}$ - 100 μg $ml^{-1}$)

**Whole genome sequencing**

Whole genome of the TS mutants was performed by next generation sequencer (Illumina Hiseq 2000).

**Phage transduction**

To 100 μl of overnight culture of the recipient bacteria (TS mutants #74 and #108), 200 μl of the donor phage (80α with truncated SA1392 and a $cm^r$ marker) was added, mixed with 3 ml of top agar [50% 0.3GL (casamino acid 3g, yeast extract 3g, NaCl 5.9g, 60% sodium lactate syrup 3.3ml, 25% glycerol 4ml per liter) and 0.75% agar] and poured to plate containing bottom agar (50% 0.3GL, 1.5% agar, and 37.5 μg $ml^{-1}$ chloramphenicol) and middle agar

(50% 0.3GL and 1.5% agar). The plates were grown at 30°C for 2-3 days. The transductants grown on the plates were isolated and MIC values of compound 363 against these transductants were determined by broth dilution assay.

**Screening for antibacterial compounds**

Two chemical libraries of synthetic compounds were screened independently for antibacterial activity against *S. aureus*; one of the chemical libraries contained a total of 400 compounds synthesized for the purpose of total synthesis of natural products in the laboratory of Synthetic Natural Products Chemistry, Graduate School of Pharmaceutical Sciences, the University of Tokyo and the other contained 103873 compounds from the open innovation center for drug discovery, the University of Tokyo.

**Toxicity tests in silkworms**

Fifth instar larvae of silkworms were injected 400 μg of each compounds through intrahaemolymph route and observed for three days. Toxicity was calculated as μg g•larva$^{-1}$.

| Strain | Characteristics |
|---|---|
| *Staphylococcus aureus* MSSA1 | MET-susceptible, clinical isolate |
| *S. aureus* RN4220 | MET-susceptible |
| *S. aureus* NCTC8325 | MET-susceptible |
| *S. aureus* Smith | MET-susceptible |
| *S. aureus* MRSA3 | OFXA, KAN, TET, ERM-resistant, clinical isolate |
| *S. aureus* MRSA4 | OFXA, KAN, CHL, CYP-resistant, clinical isolate |
| *S. aureus* MRSA6 | OFXA, FLX, KAN, TET, CYP, IM/CS-resistant, clinical isolate |
| *S. aureus* MRSA8 | OFXA, FLX, KAN, ERM, CYP, IM/CS-resistant, clinical isolate |
| *S. aureus* MRSA9 | OFXA, FLX, TET, ERM, CYP, IM/CS-resistant, clinical isolate |
| *S. aureus* MRSA11 | OFXA, KAN, ERM, CYP, IM/CS-resistant, clinical isolate |
| *S. aureus* MRSA12 | OFXA, FLX, KAN, ERM, IM/CS-resistant, clinical isolate |
| *Enterococcus feacalis* EF1 | VM-susceptible |
| *E. feacalis* EF5 | VM-resistant |
| *Streptococcus sangunius* JCM 5708 | |
| *S. pyogeneus* SS1-9 | |
| *S. pneumonia* JCSC6523 | |
| *S. agalactine* JCM5671 | |
| *Bacillus subtilis* JCM2499 | |
| *Bacillus cereus* JCM20037 | |
| *Pseudomonas aeruginosa* PAO1 | |
| *Escherichia coli* W3110 | |
| *Serratia marcences* 98-130-37 | |
| *Candida albicans* ATCC10231 | |
| *C. tropicalis* pK233 | |
| *Cryptococcus neoformans* H99 | |
| *Aspergillus niger* | |

MET: Methicillin

OFXA: Ofloxacin

TET: Tetracycline

ERM: Erythromycin

KAN: Kanamycin

CHL: chloramphenicol

CYP: Cyprofloxacin

IM/CS: Imipenem/ Cilastatin sodium

FLX: Flomoxef

VM: Vancomycin

**Table I: Microbial strains used in this study**

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