**New Approaches to Drug Discovery for Combating MRSA**

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Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major nosocomial pathogen that has developed resistance to many antibiotics. New anti-infective drugs to prevent and treat MRSA infection are required. Four assay systems were conducted to screen microbial cultures for new anti-infective compounds active against MRSA. Nosokomycins, new members of the phosphoglycolipid family, were discovered from a culture of *Streptomyces cylindracus* K04-0144 in an MRSA-silkworm infection assay. The target molecule of nosokomycins was suggested to be the transglycosylase of penicillin binding protein (PBP) involved in MRSA peptidoglycan biosynthesis. Spirohexaline, with a hexacine structure, was isolated from a fungal culture of *Penicillium brasiliannum* FKI-3368 in an enzyme assay of undecaprenyl pyrophosphate (UPP) synthase, which is needed for the synthesis and transport of GlcNAc–MurNAc–pentapeptides from the cytoplasmic membrane site to the external membrane site for peptidoglycan synthesis. Spirohexaline inhibited MRSA growth by the blockade of UPP synthase activity. Cylabdan, with a cysteine-carrying labdan skeleton, was also discovered from the nosokomyacin-producing actinomycete as a potentiator of imipenem activity against MRSA. The molecular target of cylabdan was identified as FemA, which is involved in the synthesis of a pentaglycine interpeptide bridge in MRSA peptidoglycan. Citridone A with a unique 6-6/5/5-ring system containing a rare phenyl-\(\mathbb{R}\)-furopyridone skeleton, originally isolated as a potentiator of antifungal miconazole activity, was found to inhibit MRSA yellow pigment production. These new microbial products will serve as lead compounds for developing new anti-infective drugs for combating MRSA.

Key words  microbial product; anti-infective; methicillin-resistant *Staphylococcus aureus*; screening; undecaprenyl pyrophosphate synthase; imipenem potentiator; staphyloxanthin

1. Introduction

The current increase in antibiotic resistant pathogenic microorganisms, including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin resistant *Enterococci*, and multidrug resistant Gram-negative bacilli has yielded a serious threat to public health.\(^4\) Therefore, discovery and development of novel antibiotics is in great demand. Considering the moderate state of new antibiotic development by pharmaceutical companies, however, academia can play an important role in discovering new antibiotics and providing potential leads for clinical drugs. Therefore, government should support academia in developing such potential antibiotics or leads, at least to the Phase I stage.

MRSA is a major nosocomial pathogen that has developed resistance to many antibiotics.\(^3\) It has been reported that MRSA has become resistant even to vancomycin, the last-resort antibiotic.\(^4\) This prompted us to search for new anti-infective compounds active against MRSA from microbial resources. Anti-infective compounds include those that inhibit the growth of pathogens, kill them, control their adaptation, survival or pathogenicity and potentiate antibiotic activity against such pathogens.\(^5\) On the basis of this anti-infective idea, four assay systems were conducted to screen microbial cultures for new anti-infective compounds active against MRSA. We believe natural products, including microbial metabolites, are the best source for new drug discovery.\(^5\) In this review, the rationales, assay (screening) systems, new compounds of microbial origin discovered in the screening systems, and their biological activities are described.

2. Screening for Anti-MRSA Antibiotics in Silkworm Infection Assay

Antibiotic candidates against pathogenic microorganisms are generally screened using *in vitro* assay systems, such as the traditional paper disk method and microdilution method; however, in most cases, these candidates subsequently show no therapeutic effects in *in vivo* assays using mammals such as mice, rabbits and monkeys, because the pharmacokinetics, systemic absorption and stability of candidates are poor in the mammal body. Therefore, *in vivo* studies have been required for developing clinical drugs. However, such studies have several inherent problems, including high costs, animal welfare, ethical issues and so on. To overcome these issues in the discovery of new anti-MRSA antibiotics, we introduced an *in vivo*-mimic infection assay using silkworms in the early stages of a screening program.

Our silkworm infection assay was carried out according to established methods.\(^6\) Briefly, MRSA culture was injected into the dorsal hemolymph of the fifth instar silkworm. Immediately, a test sample was injected into the hemolymph. If the sample was inactive, all the MRSA-infected silkworms died within 3 d. However, if vancomycin, an anti-MRSA agent clinically used, was injected, all silkworms survived. In the primary screening, those microbial culture samples were selected in which at least three of 5 MRSA-infected silkworms survived following injection. The screening results are sum-

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marized in Table 1, including the screening result from the paper disk assay. From 5340 culture samples, the primary hit number was 327 (6.1%) in the paper disk screening and 21 (0.4%) in the silkworm screening. Thus, we found that the hit rate is extremely low in this in vivo-mimic assay, with the expectation that we exclude false-positive or in vivo-inactive samples in the primary screening. Figure 1 shows the compounds isolated in the silkworm screening. Helvolic acid, 9,10) isolated from a fungal culture, was structurally related to fusidic acid, a clinically used antibiotic. Acremonidin C was reported to show in vivo efficacy in the mouse infection assay with MRSA. 11) Furthermore, gilvocarcin V 12) and lavanducyanin,13) originally reported as antitumor compounds, were found to show efficacy in our assay system without toxicity to the host silkworms.

From this screening program, new compounds, named nosokomycins A to D, were isolated from a culture of Streptomyces cyslabdanicus K04-0144.8) They are structurally related to moenomycins,14) which consist of a polypropenyl chain, a phosphoglycerate, and a carbohydrate chain (pentasacharide). Moenomycins were known to inhibit the transglycosylase of penicillin binding protein (PBP) involved in bacterial peptidoglycan biosynthesis,15,16) suggesting that the target molecule of nosokomycins is transglycosylase. We first confirmed that nosokomycins A and B were effective at 50 µg/larva in the silkworms infected with MRSA. Then, from the agar diffusion assay, all nosokomycins showed potent activity against Gram-positive bacteria, including multidrug-resistant MRSA17) (resistant to methicillin, imipenem, ciprofloxacin and tobramycin) with analogous minimum inhibitory concentration (MIC) values (0.125–0.25 µg/mL). Furthermore, nosokomycins B and D exhibited moderate activity against Gram-negative bacteria.18) From the population analysis using 54 clinically isolated MRSAs, nosokomycins A to D completely inhibited almost all the MRSAs at 0.50, 0.25, 1.0 and 0.50 µg/mL, respectively, while vancomycin, arbekacin and linezolid did at 1.0, 1.0 and 2.0 µg/mL, respectively.18) These findings indicated that nosokomycins are as potent as, or even more potent than, current clinically used anti-MRSA antibiotics.

From this silkworm screening, in collaboration with Sekimizu’s group, new anti-MRSA antibiotics named lysocins were discovered in the bacterial culture of Lysobacter sp. RH2180-5.19) Lysocin E showed potent in vitro activity against MRSAs and in vivo efficacy in the mouse model. Hamamoto et al. revealed that the mechanism of action lies in its specific in-
3. Screening for Inhibitors of MRSA Undecaprenyl Pyrophosphate Synthase

Undecaprenyl pyrophosphate (UPP) is a key lipid involved in the biosynthesis of bacterial peptidoglycan. In the bacterial cell wall synthetic pathway, as shown in Fig. 2, UPP is needed for the synthesis and transport of hydrophilic GlcNAc–MurNAc–pentapeptides across the hydrophobic environment of the cytoplasmic membrane to the externally located sites of polymerization. Enzymes involved in peptidoglycan synthesis are anticipated to be ideal targets for the development of novel antibiotics.

UPP synthase produces UPP by consecutive condensation reactions of one farnesyl pyrophosphate (FPP) with 8 isopentenyl pyrophosphates (IPP). UPP synthase inhibitors, therefore, will cause specific growth inhibition of bacteria including MRSA. Accordingly, an enzyme assay for UPP interaction with bacterial menaquinone. We expect that these compounds discovered in the silkworm assay will be developed as practically useful anti-MRSA drugs.
synthase was conducted using recombinant MRSA UPP synthase. The amount of pyrophosphate produced by the enzyme was determined according to the established method.\textsuperscript{21)} IPP-derived pyrophosphate was hydrolyzed by inorganic pyrophosphatase\textsuperscript{22)} to determine the amount of phosphate. As a practical screening strategy, a total of 12118 cultures (5941 actinomycetes and 6177 fungi) were first screened for antimicrobial activity against \textit{S. aureus} and \textit{B. subtilis}. As a result, 949 showed antimicrobial activity. Next, they were evaluated in the UPP synthase assay, and 123 culture samples were selected (1.0%). Finally, four compounds were purified and identified, as shown in Fig. 3. Among these, spirohexaline and \textit{epi}-trichosetin were new compounds. Spirohexaline and structurally related viridicatamtoxin were isolated from a fungal culture of \textit{Penicillium brasiliannum} FKI-3368.\textsuperscript{22)} These compounds inhibited UPP synthase activity with IC\textsubscript{50} values of 9.0 and 4.0 \(\mu\)M, and exhibited anti-MRSA activity with MICs of 1.56 and 6.25 \(\mu\)g/mL, respectively. From further selectivity studies, these were found to more selectively inhibit UPP synthase than the functionally related enzymes, octaprenyl pyrophosphate synthase from \textit{Escherichia (E.) coli} and mammalian dehydrodolichyl pyrophosphate synthase. \textit{epi}-Trichosetin (Fig. 3) was isolated, along with the known stereoisomer trichosetin, from a fungal culture of \textit{Fusarium oxysporum} FKI-4553.\textsuperscript{23)} Both \textit{epi}-trichosetin and trichosetin moderately inhibited UPP synthase activity, with IC\textsubscript{50}s of 82 and 30 \(\mu\)M, respectively, and thus exhibited anti-MRSA activity. These inhibitors will be evaluated by silkworm infection assay to define their potential for further developmental studies.

### 4. Screening for Potentiators of Imipenem Activity against MRSA

MRSA is resistant to \(\beta\)-lactam drugs, including imipenem, the most common clinically used \(\beta\)-lactam. One of our screening ideas for combatting MRSA was to search for compounds that could restore imipenem activity against MRSA.

This screening system was based on a common technique, namely, comparing the anti-MRSA activity of samples by a paper disk method in the presence and absence of imipenem to select microbial culture samples that show anti-MRSA activity only in the presence of imipenem. More than 10000 culture samples were screened, and two cultures, \textit{Aspergillus} sp. FKI-2136 and \textit{Streptomyces cyslabdanicus} K04-0144, were selected. The former fungus was found to produce new tetracyclic quinones, designated stemphones B to G\textsuperscript{24,25)} (Fig. 4). The latter strain\textsuperscript{26)} produced four new compounds: three cyslabdans with a labdan-type diterpene structure connecting to an N-acetylcysteine residue via thioether linkage,\textsuperscript{27,28)} and nosokophic acid, predicted to be a biosynthetic intermediate of moenomycins.\textsuperscript{29)} The stereochemistry of these compounds was determined in part by nuclear Overhauser effect (NOE) experiments and degradation studies. Recent study of the total synthesis of cyslabdan enabled the determination of complete relative and absolute stereochemistry.\textsuperscript{30)} As described above, nosokomycins were also isolated from the same culture of this actinomycete. Thus, \textit{Streptomyces cyslabdanicus} K04-0144 is a versatile microbe that produces three kinds of unique compounds, cyslabdans, nosokophic acid and nosokomycins, for combatting MRSA. These findings indicate the high potentiality and importance of microbial sources.

Recently, analysis of the genome sequence of \textit{S. cyslabdanicus} K04-0144 revealed that a set of four genes, \textit{cldA}, \textit{cldB}, \textit{cldC}, and \textit{cldD}, were involved in the biosynthesis of cyslabdan.\textsuperscript{31)} These gene products were found to form a key epoxy intermediate from three IPPs and one dimethylallyl pyrophosphate (DMAPP). Subsequently, an intermediate was converted into mycothiol-S-conjugate through a non-enzymatic nucleophilic reaction, which was finally hydrolyzed to generate cyslabdan. This labdan-type diterpene backbone connecting to an N-acetylcysteine residue \textit{via} thioether linkage is very rare.
among bacterial metabolites. This study provided an important biosynthetic insight into this diterpene family.

All of the compounds listed in Fig. 4 enhanced the imipenem activity against MRSA; however they themselves showed no activity against MRSA. Furthermore, a comparative study with a microdilution method indicated that cyslabdan is the most active in enhancing imipenem activity against MRSA (Table 2). Interestingly, cyslabdan had no restoration activity in combination with other typical antibiotics (streptomycin, vancomycin, tetracycline and ciprofloxacin). Furthermore, among β-lactam drugs, cyslabdan effectively restored carbapenem activity against MRSA by over 1000-fold. Similarly, the potentiation of stemphone C and nosokophic acid tended to be selective towards β-lactam drugs.

To identify the molecular target responsible for cyslabdan’s unique biological activity, cyslabdan-binding proteins were searched in an MRSA lysate, leading to the identification of FemA, which is involved in the synthesis of the pentaglycine interpeptide bridge in MRSA peptidoglycan. Furthermore, analysis of peptidoglycan accumulated in cyslabdan-treated MRSA, and the inhibition of FemA enzymatic activity by cyslabdan, supported the conclusion that the primary molecular target of cyslabdan is FemA.

Our working hypothesis for the mechanism of the synergic action of cyslabdan is illustrated in Fig. 5. When MRSA is treated with cyslabdan, FemA is inhibited, resulting in the accumulation of monoglycyl and nonglycyl murein monomers, intermediates of peptidoglycan. However, cyslabdan alone

![Fig. 5. A Working Hypothesis on the Mechanism of Synergic Anti-MRSA Action in Combination with Imipenem and Cyslabdan](image)

Four cases are shown here: a) no treatment, b) in the presence of imipenem, c) in the presence of cyslabdan, and d) combination of imipenem and cyslabdan.

Table 2. Potentiating Activity of Imipenem of Stemphones, Cyslabdans and Nosokophic Acid against MRSA

<table>
<thead>
<tr>
<th>Compound</th>
<th>MIC (µg/mL) of imipenem&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Potentiation ratio (fold)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.03</td>
<td>1</td>
</tr>
<tr>
<td>Stemphone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.03</td>
<td>533</td>
</tr>
<tr>
<td>C</td>
<td>0.03</td>
<td>533</td>
</tr>
<tr>
<td>D</td>
<td>0.06</td>
<td>267</td>
</tr>
<tr>
<td>E</td>
<td>0.03</td>
<td>533</td>
</tr>
<tr>
<td>F</td>
<td>0.06</td>
<td>267</td>
</tr>
<tr>
<td>G</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td>Cyslabdan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.125</td>
<td>128</td>
</tr>
<tr>
<td>C</td>
<td>0.03</td>
<td>533</td>
</tr>
<tr>
<td>Nosokophic</td>
<td>0.03</td>
<td>533</td>
</tr>
</tbody>
</table>

<sup>a</sup> Potentiating activity of imipenem against MRSA is evaluated by measuring the MIC value of imipenem against MRSA in the absence and presence of the compounds of 10 µg/mL.  
<sup>b</sup> Potentiation ratio is expressed as MIC value in the absence of a compound/MIC value in the presence of a compound.
has almost no effect on the growth of MRSA and methicillin-susceptible Staphylococcus aureus (MSSA), indicating that at least PBP, and possibly PBP2, recognize monoglycyl muropeptide monomers as a substrate and crosslink them to build the peptidoglycan. On the other hand, in combination with imipenem and cyslabdan, the growth of MRSA is completely inhibited, indicating that imipenem-insensitive PBP2 cannot crosslink monoglycyl and nonglycyl muropeptides, resulting in the failure of MRSA peptidoglycan formation. Importantly, this model highlights the differences of PBP and PBP2 in their substrate specificity. Accordingly, blocking the supply of the pentaglycyl substrates favorable for PBP2 would be an effective approach for combating MRSA by combination therapy with β-lactam drugs. To the best of our knowledge, cyslabdan is the first identified FemA inhibitor, which positions it as a lead compound for the development of new types of anti-MRSA drugs.

5. Screening for Inhibitors of MRSA Yellow Pigment Production

*S. aureus*, including MRSA, produces a yellow pigment called staphyloxanthin (STX). Recently STX has been recognized as one of the important virulent factors of *S. aureus*. STX, located in the cell membrane, plays an important role in enhancing the survival and infectivity of *S. aureus* in host cells. STX is composed of glucose, prenyl and fatty acyl residues. Liu *et al.* reported that a mutant that lacked the CrtM (squalene synthase) gene involved in STX production failed to survive in a mouse host. This finding indicated that inhibitors of STX production could be new anti-infective drugs against MRSA. Several known compounds, BPH-652, zaragozic acid, 7-benzyloxyindoles and flavones, were reported to inhibit STX production. Among these, BPH-652, an inhibitor of squalene synthase, proved in vivo efficacy, blocking the infection of *S. aureus* in the host mice. Based on this background, we began a search for STX inhibitors from natural sources. A new agar plate assay was conducted in which STX was produced by MRSA under specific conditions. Microbial culture sample-containing paper disks (test samples) were placed on an MRSA agar plate, and white zone-showing samples (meaning that yellow pigment (STX) production was inhibited while MRSA grew normally) were selected. A total of 45000 cultures were screened, and 27 samples (0.06%) showed a white zone. Ultimately, three types of new compounds, citridone A, graphiumins and tylopilusins (Fig. 6), were discovered.

Citridones, originally isolated as potentiators of miconazole activity against Candida albicans from the culture broth of Penicillium sp. FKI-1938, were found to inhibit yellow pigment production without growth inhibition of MRSA. Among these, citridone A was the most potent, with an IC₅₀ of 37.9 µM in the liquid culture. Citridone A has a 6-6/5/5-ring system containing a rare phenyl-R-furopyridone skeleton. Because of its unique structure and biological properties, two groups have already been subjected to total synthesis. To understand the structure–activity relationship, 11 citridone A derivatives were synthesized. Of these, three derivatives, 1, 2 and 3, showed moderate inhibition with IC₅₀ ranging from 76.8 to 101 µM (Table 3). These findings indicated that the 4,5,6a-trimethyl-4,6a-dihydro-3aH-cyclopentafuran skeleton in citridones is responsible for the inhibition of MRSA yellow pigment production.

Ten new thiodiketopiperazines graphiumins, A to J, were isolated from the culture of the marine-derived fungus Graphium sp. OPMF00224. The structures of these graphiumins, including absolute configuration, were elucidated by spectroscopic analyses and chemical methods. Graphiumins, I and J, moderately inhibited the yellow pigment production of

![Fig. 6. Microbial STX Inhibitors Discovered from Phenotype-Based Screening and Citridone A Derivatives](image-url)
MRSA, with IC₅₀ values of 80 and 894 μM, respectively (Table 3).

Diphenolic racemates, (±)-tylopiuslin A and (±)-tylopiuslin B, and tylopiuslin C, were isolated from the fruiting bodies of *Tylopius eximius*. The racemates tylopiuslins A and B, were separated into each enantiomer, and absolute configuration was assigned by electronic circular dichroism calculations. Each showed moderate inhibition of MRSA yellow pigment production by the paper disk method (Table 3).

Further experiments are needed to investigate the target molecules and in vivo efficacy of the STX inhibitors.

6. Conclusion

Microorganisms have historically provided a number of practically useful compounds, including antibiotics, cholesterol-lowering drugs, immunosuppressants and so on. It is a general understanding that microbial products are structurally diverse and thus have a potential role in the discovery of drugs with novel mechanisms of action.

Based on this belief, anti-infective drugs active against MRSA were sought from microbial metabolites in the 4 original screening systems. Phosphoglycolipid nosokomycins were discovered from an actinomycete culture as an anti-MRSA antibiotic in the in vivo-mimic screening system using silkworms. Nosokomycin A also proved in vivo activity against MRSA in the mouse infection system. Spirohexaline was discovered from a fungal culture to be an inhibitor of UPP synthase in the target-based screening system. Citridone A has a unique 6-6/5/5-ring system containing a rare phenylfuropyridone skeleton.

Thus, these microbial products have unique structures and intriguing biological activities. Furthermore, some provided new potential targets in the development of anti-MRSA drugs, and others are also expected to provide new targets for further studies on mechanisms of action. The fundamental screening studies described herein, and these microbial compounds discovered, will likely lead to the development of practically useful drugs for combating MRSA.

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Conflict of Interest

The author declares no conflict of interest.

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