ORIGINAL ARTICLE

Inhibition of bacterial undecaprenyl pyrophosphate synthase by small fungal molecules

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Viridicatumtoxin and spirohexaline, small fungal molecules with a tetracyclic scaffold and an additional spirobicyclic ring in common, were found to inhibit bacterial undecaprenyl pyrophosphate (UPP) synthase with IC_{50} values of 4 and 9 μ M, respectively. These molecules showed weak inhibitory activity against catalytically related enzymes such as bacterial octaprenyl pyrophosphate synthase and yeast dehydrolidyl pyrophosphate synthase, indicating that the compounds preferentially inhibit UPP synthase. They showed antimicrobial activity, particularly against Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA). Furthermore, molecular modeling strongly suggested that the hydrophobic spirobicyclic ring of viridicatumtoxin interacts with three hydrophobic clefts of the active site in MRSA UPP synthase.

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INTRODUCTION

Recent increases in antibiotic-resistant pathogenic organisms such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci* and multi-drug-resistant Gram-negative bacilli, coupled with the current state of new antibiotics developed by pharmaceutical companies, have created a serious threat to public health.^{1–3} Therefore, there is great demand for the discovery and development of new anti-infective agents possessing a novel mechanism of action and an enhanced antimicrobial profile.

The bacterial cell wall is an attractive target for the development of new antibacterial agents because it is an essential, bacterial-specific structure that is absent in human cells. The biosynthesis of peptidoglycan can be divided into three different stages (Figure 1).⁴ The first stage occurs in the cytoplasm and leads to the synthesis of the nucleotide sugar-linked precursor (UDP-MurNAc-pentapeptide and UDP-GlcNAc). In the second stage, precursor lipid intermediates are synthesized at the cytoplasmic membrane. The phospho-MurNAc-pentapeptide moiety of UDP-MurNAc-pentapeptide is transferred to the membrane acceptor bactoprenol, yielding lipid I [MurNAc-(pentapeptide)-pyrophosphoryl-undecaprenol]. Then, GlcNAc moiety from UDP-GlcNAc is added to lipid I, yielding lipid II [GlcNAc-MurNAc-pentapeptide-pyrophosphoryl-undecaprenol], which is the substrate for the polymerization reaction of the bacterial cell wall. At this stage, the translocation of lipid II from the cytoplasmic side to the outer side of the cytoplasmic membrane occurs. The final stage of peptidoglycan biosynthesis, which takes place at the outer side of the membrane, involves the polymerization of the newly synthesized disaccharide units and incorporation into the growing cell wall peptidoglycan with the penicillin-binding proteins that catalyze the

transglycosylation and transpeptidation reactions. All of these stages are potential targets for the development of novel antibiotics.

Undecaprenol, also referred to as a bactoprenol, is a key lipid involved in the biosynthesis of peptidoglycan and a variety of other cell-wall polysaccharide components, such as lipopolysaccharides, the enterobacterial common antigen, capsule polysaccharides, and teichoic acids in certain prokaryotes. In the cell wall biosynthetic pathway, undecaprenyl monophosphate (C55-P) is needed for the synthesis and transport of hydrophilic GlcNAc-MurNAc-pentapeptides across the hydrophobic cytoplasmic membrane to the externally located sites of polymerization (periplasm sites). The precursor for C55-P, undecaprenyl pyrophosphate (UPP), is synthesized by the addition of eight C5 isopentenyl units (*cis*[Z]-configuration) onto C15-PP (all-*trans* [E])-farnesyl pyrophosphate (FPP) (Figure 2). This reaction is catalyzed by UPP synthase (di-*trans*-poly-*cis*-decaprenyl cistransferase; EC 2.5.1.31). FPP itself results from head-to-tail condensation of isopentenyl pyrophosphate with dimethylallyl pyrophosphate, generating C10-PP geranyl pyrophosphate, followed by a second condensation of isopentenyl pyrophosphate, a reaction catalyzed by the FPP synthase that is a prototype of the *trans*-prenyltransferase family. The structure and mixed *E*, *Z* stereochemistry of the C55-prenyl product (Figure 2), as deduced from recently obtained knowledge about the reaction mechanism of UPP synthase, confirmed earlier data from MS and NMR spectrometry of the undecaprenol isolated from bacterial membranes. The X-ray crystal structures of several UPP synthases from different species (for example, *Escherichia coli*, *Micrococcus luteus* and *Helicobacter pylori*) reveal a generally conserved architecture.^{5–7} UPP synthase exists as a homo-dimer of identical subunits, each consisting of five conserved regions including isopentenyl

Figure 1 Cell wall synthesis pathway in bacteria.

Figure 2 Undecaprenyl pyrophosphate synthase reaction.

pyrophosphate binding, allylic pyrophosphate binding and essential catalytic sites.⁸ Subsequently, a structure-based approach derived from isopentenyl diphosphate^{9–11} and computer-aided drug design^{7,12–14} have been reported. However, their UPP synthase inhibitory activity did not necessarily reflect the antibacterial activity.

In the present study, as part of our interest in discovering natural small molecules that potently and selectively inhibit UPP synthase, we screened about 10 000 culture extracts from soil-isolated actinomycetes and fungi for inhibitors of *S. aureus* recombinant UPP synthase activity. Bioactivity-guided fractionation of the active crude extract from the fermentation culture of *Penicillium brasilianum* FKI-3368 using various chromatographic techniques¹⁵ led to the discovery of a new compound designated spirohexaline together with known structurally related viridicatumtoxin (Figure 3). Viridicatumtoxin was originally isolated from *P. viridicatum* and showed toxic activity against mice and rats with a single i.p. dose of 80 and 90 mg kg^{−1}, respectively.^{16–18} Recently, viridicatumtoxin B (Figure 3) was reported to show anti-microbial activity against Gram-positive bacteria including MRSA.^{19,20} However, its molecular targets have not been reported. In this study, we reveal that these compounds inhibit the UPP synthase activity of *S. aureus* with anti-MRSA activity.

MATERIALS AND METHODS

Materials

Viridicatumtoxin and spirohexaline were obtained from a culture broth of *P. brasilianum* FKI-3368.¹⁵ [1-¹⁴C]Isopentenyl pyrophosphate (IPP,

1.48 GBq mmol^{−1}) was purchased from PerkinElmer (Waltham, MA, USA). A precoated reversed-phase TLC plate, RP-18, from Merck KGaA (Darmstadt, Germany) was used. Amplex Red was from Invitrogen (Carlsbad, CA, USA). Oligonucleotides were purchased from Operon Biotechnology Co. Ltd. (Tokyo, Japan). IPP, FPP and other biochemical reagents were from Sigma-Aldrich (St Louis, MO, USA) or Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Bacterial strains, plasmid and enzymes

The *E. coli* strains used in this study were JM109 (Takara Bio Inc., Shiga, Japan) and BL21(DE3) (Novagen, Abingdon, UK), which were grown in Luria-Bertani (LB) medium (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) with aeration at 37 °C. *Saccharomyces cerevisiae* OP-3C was as described previously.²¹ Plasmid pET-42b was from Novagen. Inorganic pyrophosphatase, purine nucleoside phosphorylase, xanthine oxidase and horseradish peroxidase were purchased from Sigma-Aldrich. Potato acid phosphatase was purchased from GE Healthcare UK Ltd. (Buckinghamshire, UK). Restriction enzymes and other DNA-modifying enzymes were purchased from New England Biolabs (Beverly, MA, USA) or Takara Bio Inc. and used as described by the manufacturers.

General DNA techniques and transformation

Chromosomal DNAs of *S. aureus* and *E. coli* were prepared using the Qiagen (Hilden, Germany) genomic DNA purification system. To prepare plasmid DNA, a High Pure Plasmid Isolation Kit (Roche Diagnostics GmbH, Mannheim, Germany) was used. Plasmids, PCR products and chromosomal DNA were cleaved with the appropriate restriction enzymes, ligated and transformed into *E. coli* JM109 cells. Transformed cells were selected on LB agar plates containing kanamycin (30 µg ml^{−1}). DNA manipulations in *E. coli* were performed as described by Sambrook and Russell.²²

PCR and sequencing

PCR was carried out using a PCR Thermal Cycler Dice mini (Takara Bio Inc.). Automatic DNA sequencing was carried out using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Carlsbad, CA, USA) and analyzed on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems).

Figure 3 Structures of viridicatumtoxin and spirohexaline.

Constructions of the expression plasmids for UPP synthase and OPP synthase

UPP synthase gene from *S. aureus* and octaprenyl pyrophosphate (OPP) synthase gene from *E. coli* were expressed as untagged proteins under the control of the T7 promoter. The full-length genes were amplified by using the following forward and reverse primers: 5'-GCA TAT GTT TAA AAA GC-3' and 5'-GGG AAG CTT CTA CTC CTC ACT C-3' for the UPP synthase gene and 5'-GCA TAT GAA TTT AGA AAA AAT CAA TGA G-3' and 5'-GTG GAA GCT TAA CGA TCG CGT TGA ACA G-3' for the OPP synthase gene, respectively. The forward primer used introduced an *Nde*I site overlapping the potential methionine starting codon and the reverse primer a *Hind*III site downstream of the stop codon. The PCR products were digested with *Nde*I and *Hind*III and cloned in pET-42b digested with *Nde*I and *Hind*III.

Overexpression and preparation of UPP synthase and OPP synthase

The plasmids with correct UPP synthase or OPP synthase gene sequences were transformed into *E. coli* BL21 (DE-3). As single transformant was grown overnight at 27 °C in LB containing kanamycin (30 µg ml⁻¹). The 2 ml overnight cultures were used to inoculate 200 ml of fresh LB medium containing kanamycin (30 µg ml⁻¹) and allowed to grow to OD₅₉₀ = 0.4 before induction with 1 mM IPTG (final concentration). The cultures were incubated for 18 h at 27 °C and harvested by centrifugation. Cell pellets were frozen and stored at -70 °C before use.

Enzyme purification was carried out at 4 °C or on ice. The cell culture (200 ml) was collected to yield approximately 2 g of cell paste, which was suspended in 2 ml of lysis buffer containing 100 mM Tris (pH 7.5) and Complete Protease Inhibitor Cocktail (Roche Diagnostics GmbH). A French pressure cell press apparatus (AIM-AMINCO Spectronic Instruments, Lake Forest, NY, USA) was used to break the cells at 10 000 psi. The cell lysate was centrifuged at 100 000 × g for 1 h and the supernatant was used for enzyme sources.

Preparation of microsomal fraction of *S. cerevisiae*

Yeast microsomal fraction was used as the source of dehydrodolichyl pyrophosphate (DedolPP) synthase. *S. cerevisiae* OP-3C was grown at 30 °C in 100 ml of minimal medium containing 2% glucose and 0.67% yeast nitrogen base w/o amino acid (Becton, Dickinson and Company Japan, Tokyo, Japan) and 0.5% casamino acids (Becton, Dickinson and Company Japan) and 0.04% adenine until the late logarithmic phase (OD₅₉₀ of 1.5). The cells were harvested by centrifugation at 5000g for 10 min, suspended in 10 ml of buffer A containing 10 mM Tris-HCl (pH 7.5), 1.2 M sorbitol, 1 mM dithiothreitol, 1 mM EDTA and 0.5 mg ml⁻¹ zymolyase 100 T (Seikagaku Corp., Tokyo, Japan) and incubated at 30 °C for 10 min. The spheroplast-formed cells were collected by centrifugation at 1000g for 5 min and resuspended in 2 ml of buffer B containing 20 mM

Tris-HCl (pH 7.5), 2 mM MgCl₂, 1 mM dithiothreitol and one tablet of Complete Protease Inhibitor Cocktail Tablet (GE Healthcare UK Ltd) per 10 ml and homogenized with a Potter-Elvehjem tissue grinder in an ice-water bath for 30 s. Cell lysates were centrifuged at 12 000g for 20 min to remove unbroken cells. Supernatants were further centrifuged at 100 000g for 1 h to prepare the microsome-containing fraction.

Assay for UPP synthase and OPP synthase

UPP synthase reaction was carried out according to the established method with some modification.²³ Reactions were performed in black 96-well plates from DS Pharma Biomedical Co., Ltd. (Osaka, Japan). The partially purified enzyme fraction containing UPP synthase or OPP synthase was added to the reaction mixture (100 µl) containing 100 mM Tris-HCl (pH 7.5), 0.5 mM MgCl₂, 50 mM KCl, 3.5 µM IPP, 0.5 µM FPP, 0.005% (w/v) Triton X-100 and 5 mU of inorganic pyrophosphatase from *S. cerevisiae* in a total volume of 100 µl. The reaction was carried out at 37 °C, terminated after 30 min by the addition of 0.5 M EDTA (10 µl) and quenched with a cocktail (100 µl) containing 100 mM Tris-HCl (pH 7.4), 3 mM inosine, 0.2 mU ml⁻¹ purine nucleoside phosphorylase, 10 mU ml⁻¹ horseradish peroxidase, 4 mU ml⁻¹ xanthine oxidase and 0.1 mM Amplex Red. After another 30-min incubation at room temperature, the plate was read at 530/590 nm. The amount of phosphate released was calculated from a standard curve prepared with KH₂PO₄.

Analysis of products generated by UPP synthase, OPP synthase and DedolPP synthase

These polyprenyl pyrophosphate (UPP, OPP and DedolPP) synthase activities were measured by determining the amounts of [1-¹⁴C]IPP incorporated into butanol-extractable polyprenyl pyrophosphates.²⁴ The standard assay was carried out in a reaction mixture (200 µl) containing 100 mM Tris-HCl (pH 7.5), 0.5 mM MgCl₂, 10 µM FPP, 10 µM [1-¹⁴C]IPP (37 MBq mol⁻¹, GE Healthcare, UK Ltd), 0.005% (w/v) Triton X-100 and a suitable amount of each individual enzyme solution (UPP synthase, OPP synthase or DedolPP synthase). The reaction was initiated by the addition of an enzyme solution. After incubation at 37 °C for 30 min, the reaction products were extracted with 1-butanol saturated with water and hydrolyzed to the corresponding alcohols with potato acid phosphatase as described by Koyama *et al.*²⁵ The alcohols were extracted with 1-pentane and then developed by reversed-phase TLC (Merck KGaA) with a solvent system of acetone/water (19:1) for UPP synthase and OPP synthase, or acetone/water (39:1) for DedolPP synthase. The TLC plates were exposed to Fuji imaging plates at room temperature for 1 day, and then analyzed with an FLA-7000 Bioimage Analyzer (GE Healthcare, UK Ltd).

Homology modeling of UPP synthase and UPP synthase-spirohexaline complexes

Model structures of UPP synthase were constructed based on the homology modeling method.²⁶⁻²⁸ Reference proteins and sequence alignments were searched using PSI-BLAST. The amino acid sequence of *S. aureus* UPP synthase (accession no. Q6GHH5_STAAR) was used as a target protein. The X-ray structures of *E. coli* UPP synthase (PDB code: 1F75_A) were defined and used as a reference protein. The sequence alignments produced by PSI-BLAST were manually adjusted on the biologically important regions and the secondary structure using the CHIMERA modeling system.^{26,27} Then, a homodimer model of UPP synthase-UPP complex was constructed using the computer software FAMS Ligand & Complex based on these alignments. Docking models of UPP synthase with an inhibitor were simulated using ChooseLD.²⁸

Antimicrobial assay

MIC was determined by the agar dilution method²⁹ using Difco Potato Dextrose Agar (Becton, Dickinson and Company Japan) and Difco Mueller Hinton Agar (Becton, Dickinson and Company Japan) for antifungal and antibacterial tests, respectively. Freshly grown cells (or spores) of 16 test microorganisms were inoculated and grown at 25 °C for fungi and 37 °C for bacteria.

Cytotoxic assay

The cytotoxicity of a sample to Jurkat cells was measured by colorimetric assay using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (Sigma-Aldrich).³⁰ Jurkat cells (5×10^5 cells in 100 μ l) were added to each well of a 96-well microplate. A sample (1 μ l in MeOH) was added to each well to make a final concentration of 0–92 μ M. The cells were incubated for 6 h at 37 °C. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (10 μ l of 5.5 mg ml⁻¹ stock solution) and lysis solution (90 μ l, 40% *N,N*-dimethylformamide, 20% sodium dodecyl sulfate, 2% acetic acid and 0.03% HCl) were added to each well, and the microplate was shaken for 2 h. The optical density of each well was measured at 540 nm using a microtiter-plate reader (Elx 808; BioTek Instruments, Winooski, VT, USA).

Other analytical methods

Protein concentration was determined using a BCA protein assay kit (Thermo Fisher Scientific Inc., Waltham, MA, USA).

RESULTS

Inhibition of UPP synthase by viridicatumtoxin and spirohexaline

Viridicatumtoxin and spirohexaline produced by *P. brasilianum* FKI-3368 were discovered as inhibitors of bacterial UPP synthase activity through our screening program.¹³ In this study, the effects of these molecules on the activity of recombinant *S. aureus* UPP synthase were first investigated by an enzyme-coupled fluorescent method. As shown in Figure 4a, they dose-dependently inhibited UPP synthase activity with IC₅₀ values of 4 and 9 μ M, respectively. Next, the effects of

Figure 4 (a) Inhibition of undecaprenyl pyrophosphate synthase and octaprenyl pyrophosphate synthase by viridicatumtoxin and spirohexaline. Closed character, UPP synthase; open character, OPP synthase; circle, viridicatumtoxin; triangle, spirohexaline. (b) Effect of preincubation of UPP synthase activity with viridicatumtoxin. UPP synthase was preincubated with (closed triangle) or without (closed circle) viridicatumtoxin.

Figure 5 Effects of viridicatumtoxin on the synthesis of polyprenyl alcohols by UPP synthase, OPP synthase and DeDoIPP synthase. (a) Autoradiography after separation of respective prenyl alcohols C55, C40 and C80-90 on THILC by reactions of UPP synthase, OPP synthase and DeDoIPP synthase in the presence of viridicatumtoxin (0(control) – 100 μ M). (b) Inhibition of polyprenyl alcohols production by viridicatumtoxin. From the data (a), inhibition % (sample product/control product $\times$ 100) was calculated. (c) IC₅₀ values for the three enzymes calculated from the data (b).

the two molecules on *E. coli* OPP synthase, an analogous enzyme that catalyzes the *trans*-type condensation reaction of FPP with IPP to generate *trans*-OPP, was investigated by a similar enzyme-coupled fluorescence assay. Viridicatumtoxin and spirohexaline inhibited OPP synthase activity with higher IC_{50} values of 16.4 and 64.6 μM , respectively (Figure 4a).

As viridicatumtoxin was more potent than spirohexaline in UPP synthase inhibition, it was selected for further experiments. In order to confirm the potency and selectivity, the amounts of the respective reaction products, [^{14}C]C55, [^{14}C]C40 and [^{14}C]C80-90 from [^{14}C] IPP produced by UPP synthase, OPP synthase and DedolPP synthase, in the presence or absence of viridicatumtoxin, were analyzed by TLC. As shown in Figure 5a, [^{14}C]C55 was detected as a major product by UPP synthase (left column, control). As expected, the radioactivity of [^{14}C]C55 was dose-dependently decreased in the presence of the inhibitor, and completely disappeared at 10 and 100 μM . The IC_{50} value was calculated to be 3.1 μM . On the other hand, OPP synthase, which produced *trans*-OPP as a major product DedolPP, was inhibited by viridicatumtoxin with IC_{50} of 21 μM . Furthermore, DedolPP synthase, which produced dehydrololichyl pyrophosphate, was inhibited by viridicatumtoxin with an IC_{50} of 71 μM . These data indicate that viridicatumtoxin rather selectively inhibited UPP synthase among the catalytically related synthases.

Figure 6 Dixon plot of the inhibition of UPP synthase by viridicatumtoxin.

Table 1 Antimicrobial activities of viridicatumtoxin and spirohexaline

Microorganisms	MIC ($\mu g\ ml^{-1}$)	
	1	2
<i>Staphylococcus aureus</i> ATCC6538p (MSSA)	0.78	1.56
<i>Staphylococcus aureus</i> K24 (MRSA)	0.78	6.25
<i>Bacillus subtilis</i> ATCC6633	0.4	<0.2
<i>Micrococcus luteus</i> ATCC9341	1.56	3.12
<i>Clostridium perfringens</i> ATCC3624	0.39	1.56
<i>Bacteroides fragilis</i> ATCC23745	0.78	1.56
<i>Mycobacterium smegmatis</i> ATCC607	>100	25
<i>Escherichia coli</i> NIHJ	>100	>100
<i>Klebsiella pneumoniae</i> ATCC10031	>100	>100
<i>Pseudomonas aeruginosa</i> IFO3080	>100	>100
<i>Serratia marcescens</i> IAM1021	>100	>100
<i>Candida albicans</i> ATCC64550	6.2	25
<i>Saccharomyces cerevisiae</i> ATCC9763	12.5	100
<i>Mucor racemosus</i> IFO4581	25	100
<i>Aspergillus niger</i> ATCC9642	12.5	100
<i>Penicillium chrysogenum</i> IAM12842	6.2	50

To shed light on the inhibitory mechanism of viridicatumtoxin, UPP synthase was preincubated with several concentrations of viridicatumtoxin (1, 10 and 100 μM) for 10 min at room temperature, and then diluted (final concentrations: 0.1, 1 and 10 μM , respectively) with the reaction cocktail to start the reaction of UPP synthase. The inhibition curve (Figure 4b) was dependent on the final concentration of the inhibitor, indicating that the UPP synthase was reversibly inhibited by viridicatumtoxin. Furthermore, steady-state kinetic analysis of the inhibitor was carried out (Figure 6). The Dixon plot indicated that viridicatumtoxin inhibits UPP synthase competitively with respect to the substrate FPP, and the inhibitor constant (K_i) value was calculated to be 1.8 μM .

Antimicrobial activity of viridicatumtoxin and spirohexaline

The MIC values of the two UPP synthase inhibitors against 16 microorganisms are summarized in Table 1. Viridicatumtoxin and spirohexaline showed very similar antimicrobial spectra. They were active against Gram-positive bacteria, fungi and yeasts. However, viridicatumtoxin was generally more potent than spirohexaline. Importantly, viridicatumtoxin showed the same MIC value of 0.78 $\mu g\ ml^{-1}$ (1.38 μM) against clinically isolated MRSA and laboratory used MSSA.

The cytotoxic effects of viridicatumtoxin and spirohexaline on Jurkat cells were tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Their IC_{50} values were 4.92 and 44.3 μM , respectively.

Modeling of UPP synthase/viridicatumtoxin complex

To visualize the complex between UPP synthase and viridicatumtoxin or spirohexaline, a complex model of homodimer UPP synthase-substrate FPP was constructed using FAMS Ligand Complex,³¹ a fully automated modeling system for protein ligands. Then, docking models of UPP synthase-viridicatumtoxin and UPP synthase-spirohexaline were simulated using ChooseLD.²⁸ From the docking model (Figure 7), it was predicted that viridicatumtoxin and spirohexaline bind to the substrate active center of UPP synthase. The docking model indicated that the hydrophobic cyclohexene moieties of viridicatumtoxin and spirohexaline interact with Ile57, Leu95, Phe99 and Phe148, which form the hydrophobic cleft. The hydrophilic 12-ketone moieties or 12 α -hydroxy moieties of viridicatumtoxin and spirohexaline may form a hydrogen bond with Asn35 in the structural P-loop motif, which is the binding region of pyrophosphate of the natural substrate FPP of UPP synthase. Furthermore, the 10-hydroxy moieties of the compounds may form a hydrogen bond with Met54, while 5-hydroxy moieties may form a hydrogen bond with Ala76.

DISCUSSION

Two small fungal molecules, viridicatumtoxin and spirohexaline, were discovered as inhibitors of bacterial UPP synthase through our screening program. In the present study, their inhibitory activity against catalytically related polyprenyl pyrophosphate synthases (Figures 4 and 5) and their antimicrobial activity (Table 1) were investigated. Some of the data are summarized in Table 2. First, the selectivities of the two molecules in the inhibition of recombinant UPP synthase from *S. aureus* and recombinant OPP synthase from *E. coli* were compared. From the IC_{50} values to UPP synthase, it was confirmed that viridicatumtoxin (4 μM) is more potent than spirohexaline (9 μM). The two molecules also inhibited *E. coli* OPP synthase with higher IC_{50} values (16 and 65 μM , respectively). In terms of the principle of these two enzyme assays, pyrophosphate produced by the enzyme reaction is hydrolyzed by pyrophosphatase to generate

Figure 7 Binding models in the homology model of *S. aureus* UPP synthase from docking for viridicatumtoxin (a) and spirohexaline (b). A full color version of this figure is available at *The Journal of Antibiotics* journal online.

phosphate, which is quantified by the enzyme-coupled fluorescent method. The two molecules showed no effect on the pyrophosphatase and the phosphate quantification method. Importantly, the two molecules exhibited anti-*S. aureus* activity with MIC values of $0.78 \mu\text{g ml}^{-1}$ ($1.38 \mu\text{M}$) and $1.56 \mu\text{g ml}^{-1}$ ($2.71 \mu\text{M}$), respectively. These good correlations between UPP synthase inhibition and anti-*S. aureus* activity indicated that inhibition of UPP synthase activity by the molecules caused growth inhibition of *S. aureus*. Viridicatumtoxin and spirohexaline showed no anti-*E. coli* activity, although they inhibited UPP synthase activity with even higher IC_{50} values. It might be that the molecules cannot penetrate the porins localizing in the outer membrane of *E. coli*. To confirm the correlation, the effects of viridicatumtoxin on incorporation of radiolabeled precursors ([methyl- ^3H]thymidine, [5, 6- ^3H]uracil, [4, 5- ^3H]L-leucine, and [1- ^3H]N-acetyl-D-glucosamine) into the respective macromolecules (DNA, RNA, protein and peptidoglycan) were investigated. These [^3H] precursors were added to the middle log-phase of *S. aureus* (6 ml)

in the presence and absence of viridicatumtoxin (0.9, 1.8 and $5.4 \mu\text{M}$) and incubated at 37°C for 1 h. Unfortunately, viridicatumtoxin showed no effects on the synthesis of all the macromolecules. It might be that 1 h incubation is too short to examine the macromolecule synthesis. C-55P is supplied by two pathways: the *de novo* pathway from farnesyl-PP and isoprenyl-PP by UPP synthase at the cytosolic side of the plasma membrane, and the UPP recycling pathway by its re-use from the lipid cycle at the outer side of the plasma membrane (Figure 1). Therefore, C-55P levels might gradually reduce, and it might take a long time to reach the threshold to damage MRSA growth. Otherwise, UPP may play a unknown role in MRSA growth, or an off-target of viridicatumtoxin may be involved. Viridicatumtoxin and spirohexaline showed relatively weak inhibition against yeast DedolPP synthase. Thus, the cytotoxicity of viridicatumtoxin and spirohexaline on Jurkat cells might be due to an off-target effect.

As viridicatumtoxin is a more potent inhibitor than spirohexaline, further study was carried out using this molecule. To confirm the

Table 2 Summary of biological activity of viridicatumtoxin and spirohexaline

Compound	Assay	<i>S. aureus</i>	<i>E. coli</i>	<i>S. cerevisiae</i>
		UPP synthase	OPP synthase	DedolPP synthase
Viridicatumtoxin	IC ₅₀ (μM)			
	Enzyme	4.0	16	nt
	Product	3.1	21	71
	MIC (μM)	1.38	> 177	22.1
Spirohexaline	IC ₅₀ (μM)			
	Enzyme	9.0	65	nt
	Product	nt	nt	nt
	MIC (μM)	2.71	> 177	177

Abbreviation: nt, not tested.

selectivity toward UPP synthase activity, the products generated by the three polyprenyl pyrophosphate synthases were analyzed, and the IC₅₀ values are shown in Table 2. By this assay, viridicatumtoxin rather selectively inhibited UPP synthase over OPP synthase of *E. coli* and DedolPP synthase of *S. cerevisiae*. Considering that viridicatumtoxin showed anti-*S. cerevisiae* activity, these data appeared reasonable to explain the viridicatumtoxin action.

As a key tool in structural molecular biology, a molecular docking approach was applied to propose a working hypothesis of the inhibitory mechanism between the two inhibitors and bacterial UPP synthase. The crystallographic structures of UPP synthase from *E. coli*,⁶ *M. luteus*⁵ and *H. pylori*⁷ were reported. On the basis of the sequence alignments between *E. coli* UPP synthase and *S. aureus* UPP synthase, the 3D structural model was constructed using the homology modeling program FAMS,³¹ and then the docking models of UPP synthase-viridicatumtoxin/spirohexaline were constructed using ChooseLD.²⁸ As shown in Figure 6, viridicatumtoxin and spirohexaline were well fitted into a large cleft on the molecular surface of UPP synthase. An overall model for the substrate binding sites and catalytic center in *M. luteus* BP 26 UPP synthase⁸ has been proposed. This model indicates a critical function of the structural P-loop motif as well as Asp29 and Arg33 in the binding of pyrophosphate moiety of the natural substrate FPP. The co-crystal structure of *E. coli* UPP synthase with FPP in the active site of the enzyme showed that the pyrophosphate head of FPP is bound to the backbone imine moiety of Gly29 and Arg30 as well as the side chains of Asn28, Arg30 and Arg39 through hydrogen bonding and that the hydrocarbon moiety of FPP interacts with the side chains of hydrophobic amino acids, Leu85, Leu88 and Phe89, which form the hydrophobic cleft.³² Viridicatumtoxin and spirohexaline have similar distributions of the amphipathic polarity with FPP; the cyclohexene moiety of the compounds is hydrophobic and the naphthacene skeleton of the compounds is hydrophilic. The docking models of *S. aureus* UPP synthase and viridicatumtoxin/spirohexaline indicated that the 12α-hydroxy moiety of the compounds is bound to Asn35 in a P-loop via hydrogen bonding and the hydrophobic cyclohexene moiety interacts with Ile57, Leu95, Phe99 and Phe148 on the hydrophobic cleft region. Among them, Leu95 and Phe99 in *S. aureus* UPP synthase correspond to Leu91 and Phe95 in *M. luteus* UPP synthase and Leu85 and Phe89 in *E. coli* UPP synthase, indicating that these compounds compete with FPP at the FPP binding site. The steady-state kinetics also supported this hypothesis. Thus, viridicatumtoxin and spirohexaline bind to the

active center of UPP synthase and hinder the formation of UPP synthase-IPP intermediate.

Recently, UPP synthase has been recognized as a potential target for the development of new antimicrobial agents. Accordingly, several screening systems have been developed to search for new UPP synthase inhibitors: enzyme-based assays by measuring the amounts of phosphate released in the enzyme reaction,¹⁵ as our group utilized; cell-based assays by measuring [¹⁴C]UPP generated from [¹⁴C]IPP by the enzyme; and *in silico* virtual assays in which pharmacophore models are applied from the co-crystal structural data between UPP synthase and the substrate IPP. As a result, a number of inhibitors were successfully discovered using UPP synthase of *E. coli*,¹⁰ *S. pneumoniae*,^{11,14} *H. pylori*⁷ and *S. aureus*¹² as an enzyme source. Among them, however, only a few compounds such as tetramic acid derivative 4a,¹¹ spirohexaline,¹⁵ viridicatumtoxin,¹⁵ *epi*-trichosetin,³³ biphenyl compound¹³ and isoxazole compound¹⁴ were reported to show antimicrobial activity due to the inhibition of UPP synthase, while a number of inhibitors exhibited no antimicrobial activity. In particular, a biphenyl compound is the most potent inhibitor of MRSA UPP synthase (IC₅₀ ~ 100 nM) with growth inhibitors of MRSA.¹³

In conclusion, the present work clarified the inhibitory mechanism of viridicatumtoxin and spirohexaline with UPP synthase. Further discovery of more potent inhibitors is expected.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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