ORIGINAL ARTICLE

Spirohexalines, new inhibitors of bacterial undecaprenyl pyrophosphate synthase, produced by *Penicillium brasilianum* FKI-3368

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An enzyme assay for bacterial undecaprenyl pyrophosphate (UPP) synthase was performed to screen microbial culture broths for inhibitors of UPP synthase. During the course of this screening program, an EtOH extract of a rice culture of *Penicillium brasilianum* FKI-3368 was found to inhibit UPP synthase activity. From activity-guided purification, a new compound-designated spirohexaline was isolated together with the structurally related and known viridicatumtoxin by ethyl acetate extraction silica gel and octadecylsilane column chromatographies and high-performance liquid chromatography. The structure of spirohexaline was elucidated by spectroscopic analysis, including NMR. Spirohexaline and viridicatumtoxin have a common hexacycline structure produced by fusion of a tetracycline-type ring with a spiro-type ring. They inhibited UPP synthase activity with IC₅₀ values of 9.0 and 4.0 μ M, respectively.

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INTRODUCTION

Antibiotic-resistant bacterial infections pose a major threat to public health. Methicillin-resistant *Staphylococcus aureus* (MRSA) is known as a nosocomial pathogen that has also developed resistance to many other antibiotics. Moreover, MRSA resistance to the last resort antibiotic, vancomycin, has been reported.¹ These facts suggest that *S. aureus* will acquire full resistance to vancomycin in the near future. However, the past 30 years have seen only two new classes of antibiotics introduced into clinical use, represented by the natural product daptomycin and the synthetic oxazolidinone linezolid. Therefore, there is great need for the discovery and development of new anti-infective agents possessing a novel mechanism of action.

The bacterial cell wall is an attractive target for antibacterial agents because it is an essential bacteria-specific structure that is absent from human cells.²

Undecaprenyl pyrophosphate (UPP) is a key lipid involved in the biosynthesis of peptidoglycan and other cell wall polysaccharide components, such as lipopolysaccharides, enterobacterial common antigen, capsule polysaccharides and teichoic acids. UPP-linked saccharides are also used for *N*-linked protein glycosylation that occurs in certain prokaryotes. In the cell wall synthetic pathway, 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. Thus, UPP synthase inhibitors that cause specific growth inhibition against bacteria, including MRSA or vancomycin-resistant enterococcus, are anticipated to be useful clinical drugs because such inhibitors have yet to be used in clinical or agricultural fields.

Against this background, a screening system was established to search for UPP synthase inhibitors. We used it to discover a novel compound-designated spirohexaline (1) (Figure 1) from a culture of *Penicillium brasilianum* FKI-3368, statically fermented in a rice-based medium together with viridicatumtoxin (2). In this study, we describe the taxonomy of the producing fungus FKI-3368, the production, isolation and structural elucidation of 1 and 2.

RESULTS

Taxonomic studies

Colonies on Czapek yeast extract agar (CYA) after 7 days at 25 °C (Figure 2a) were 50–55 mm in diameter, velutinous, radially sulcate, plane, with pale yellow (1 ca) aerial mycelium. The surface was covered with olive gray (1 ig) conidia, and the exudate was sparse and clear drops. The reverse side was pale yellow brown (2 ic), with an entire margin, without soluble pigment. Colonies on malt extract agar after 7 days at 25 °C (Figure 2b) were 55–57 mm in diameter, velutinous to floccose, plane, with white mycelium, covered with mistletoe gray (24 1/2 ih) conidia. The reverse side was light olive gray

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(1 1/2 ec), with an entire margin, without soluble pigment. Colonies on 25% glycerol nitrate agar after 7 days at 25 °C (Figure 2c) were 17–18 mm in diameter, velutinous, plane, with white mycelium covered with moss gray (22 ih) conidia and lacking exudate; the reverse side was dull yellow (1 lc), with an entire margin, without soluble pigment. Colonies on CYA after 7 days at 37 °C were 19–20 mm in diameter, velutinous, radially sulcate and pulvinate. The surface was covered with white floccose aerial mycelium without exudate. The reverse side was ivory (2 db), with an entire margin, without soluble pigment. Colonies on CYA at 5 °C showed no growth. Conidiophores on malt extract agar were borne on a basal felt or directly from the agar, stipes were simple, $50\text{--}250 \times 1.0\text{--}2.5 \mu\text{m}^2$ and usually rough walled. Penicilli are typically biverticillate (Figure 2d). Metulae were in verticils of 2–5, $7\text{--}9 \times 2.5\text{--}3.5 \mu\text{m}^2$, and rough walled. Phialides were ampulliform, 2–4 per metula, $6.5\text{--}7.5 \times 2.5\text{--}3.5 \mu\text{m}^2$, collula 0.5–1.5 μm wide. Conidia, borne in chains, were globose to ellipsoidal, rough, $2.0\text{--}3.0 \times 2.0\text{--}2.5 \mu\text{m}^2$ (Figure 2e). From the above

Figure 1 Structures of spirohexaline and viridicatumtoxin.

morphological characteristics, strain FKI-3368 was classified in the genus *Penicillium*, subgenus *Furcatum*, section *Furcatum* and series *Oxalica*. Moreover, from the yellow reverse color on CYA and ellipsoidal conidia, this strain corresponded to *P. brasilianum* Bat.^{3,4}

In a BLAST search using the Blastn program from the National Center for Biotechnology Information (NCBI),⁵ the nucleotide sequences of the internal transcribed spacer (ITS), beta-tubulin gene and calmodulin gene from FKI-3368 showed 99–100% similarity with those of *P. brasilianum* CBS 253.55 (ex-type) (GenBank accession numbers GU981577, GU981629 and AB667857).

From these results, the producing strain FKI-3368 was identified as *P. brasilianum* and was deposited at the International Patent Organism Depository, National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan, as FERM P-21369.

Fermentation and purification of spirohexaline (1) and viridicatumtoxin (2)

P. brasilianum FKI-3368 was cultured statically in 500-ml Erlenmeyer flasks, each containing 50 g of Italian rice and 0.5 g of seaweed powder at 27 °C for 15 days. The fermentation materials containing the mycelium (2 kg) were extracted with 50% ethanol (2l). After the ethanol extracts were filtered and concentrated to remove ethanol, the aqueous residue was extracted with ethyl acetate. The extracts were dried over Na₂SO₄ and concentrated *in vacuo* to dryness to yield crude materials (15.9 g). The materials were subjected to silica gel column chromatography (70 × 190 mm), and eluted stepwise with 100:0, 99:1, 98:2, 96:4, 90:10 and 0:100 (v/v) of CHCl₃-CH₃OH solvents (2l each). The 99:1 and 98:2 fractions (CHCl₃-CH₃OH) showing the activity were concentrated to give a dark yellow powder (10.2 g). The powder was subjected to octadecylsilane column chromatography (70 × 120 mm, Senshu Sci. Co. Ltd., Tokyo Japan), followed by stepwise elution with 40, 50, 60, 70, 80 and 90% CH₃CN (1l each). The active fractions eluted from 60 and 70% CH₃CN were combined and concentrated to give enriched 1 and 2 as yellow

Figure 2 Morphological characteristics of *P. brasilianum* FKI-3368. (a) Photograph of colonies grown on CYA at 25 °C for 7 days. (b) Photograph of colonies grown on malt extract agar at 25 °C for 7 days. (c) Photograph of colonies grown on 25% glycerol nitrate agar at 25 °C for 7 days. (d) Scanning electron micrograph of penicillas of strain FKI-3368 grown on SEM. (e) Scanning electron micrograph of conidia of strain FKI-3368 grown on SEM. Bar represents 10 μm . A full color version of this figure is available at *The Journal of Antibiotics* journal online.

powder (631 mg). The yellow powder was finally purified with preparative high-performance liquid chromatography (column, PEGASIL ODS (Senshu Sci. Co. Ltd.), 20 × 250 mm; solvent, 60% CH₃CN containing 0.05% H₃PO₄; detection, UV at 280 nm; flow rate, 7.0 ml min⁻¹). Under these conditions, **1** and **2** were eluted as peaks with retention times of 62 and 30 min, respectively. Each fraction was pooled, concentrated and extracted with ethyl acetate, which was concentrated *in vacuo* to dryness to give pure **1** (11.7 mg) and **2** (276 mg) as yellow materials.

Structural elucidation of spirohexaline (**1**)

The physicochemical properties of spirohexaline (**1**) are summarized in Table 1 and the data of **2** also shown in Table 1 are comparable with those of viridicatumtoxin as previously reported. In addition, independent interpretation of the ¹H- and ¹³C-NMR spectra together with the ¹H–¹H COSY, DEPT, HMQC and HMBC spectra led to the identification of **2** as viridicatumtoxin.⁶

Compound **1** had absorption maxima at 239 and 281 nm in the UV–visible spectrum. The IR absorption at 3438 and 1625 cm⁻¹ suggested the presence of hydroxyl and carbonyl groups in the structure. These data of **1** are similar to those of **2**, indicating that they have the same skeleton. The molecular formula of **1** was determined as C₃₁H₃₂O₁₀ on the basis of the HRESI-MS measurement of *m/z* of 563.1900, calcd 563.1917 for C₃₁H₃₁O₁₀ (M-H)⁻, indicating that in **1**, a nitrogen atom is replaced by one carbon atom and one hydrogen atom. The ¹H- and ¹³C-NMR spectral (in chloroform-*d*₁) data are shown in Table 2. The ¹³C-NMR spectrum of **1** showed 31 signals, which were classified into four methyl carbons, one methoxy carbon, four methylene carbons, one oxygenated sp³ methine carbon, two sp² methine carbons, two sp³ quaternary carbons, two oxygenated sp³ quaternary carbons, eight sp² quaternary carbons, four oxygenated sp² quaternary carbons and three ketone carbonyl carbons by analysis of DEPT spectra (Table 2). These spectral data of **1** were similar to those of **2**. The major difference between **1** and **2** in ¹H- and ¹³C-NMR data is that one methyl carbon (δ_C 27.7, δ_H 2.73) was newly observed in **1** instead of the NH₂ (δ_H 5.92, 9.08) in **2**. In addition, the amino carbonyl carbon of C-13 of **1** was downfield-shifted from δ 172.7 to δ 202.2. These spectral data together with the molecular formula suggested that a NH₂ group replaced a methyl group at C-13. Furthermore, the presence of the methyl group at C-13 was determined by the HMBC spectrum (Figure 3). The methyl proton at δ 2.73 (H₃-21) was long-range-coupled to the sp² quaternary carbon at C-2 (δ 110.5) and the

ketone carbonyl carbons at C-13 (δ 202.2), indicating that the methyl group is present at C-13. The remaining structure was also confirmed by ¹H–¹H COSY and HMBC spectra. Thus, the structure of **1** was assigned as shown in Figure 1.

Inhibition of UPP synthase and antimicrobial activities

UPP synthase activity was measured by the enzyme-coupled fluorescent method.⁷ Compounds **1** and **2** inhibited UPP synthase activity with IC₅₀ values of 9.0 μM and 4.0 μM, respectively (Figure 4). Compound **1** exhibited potent antimicrobial activity against *S. aureus* and clinically isolated MRSA with minimal inhibitory concentration values of 1.56 μg ml⁻¹ and 6.25 μg ml⁻¹, respectively.

DISCUSSION

As described in this study, spirohexaline and viridicatumtoxin (Figure 1) were isolated from the culture broth of *P. brasilianum* FKI-3368. They are potent inhibitors of bacterial UPP synthase. Furthermore, as we expected, spirohexaline was found to show potent antibacterial activity against Gram-positive bacteria, including MRSA, as described in an accompanying study. UPP synthase is an attractive target for the development of a new type of antimicrobial agents. Indeed, high-throughput screening for UPP synthase was performed by several groups.^{8–10} The Novartis group reported that tetramic acid derivatives were potent inhibitors of *Staphylococcus pneumonia* UPP synthase, and Liang's group obtained sulfonylbis-containing synthetic compound (BTB06061) as a potent and selective inhibitor of *Helicobacter pylori* UPP synthase. Furthermore, from the analysis of the X-ray structures of UPP synthase-substrate (for example, farnesyl diphosphate)/inhibitors (for example, bisphosphonate drugs) complexes and the computational modeling of substrate/inhibitors of UPP synthase, more potent inhibitors have been developed.^{11–13} However, even though such compounds inhibited UPP synthase, their antimicrobial activity was not described in most cases.^{8,10–13} It might be that the inhibitory activity against UPP synthase cannot reflect the antimicrobial activity. Among the UPP synthase inhibitors reported so far, tetramic acid derivative **4a** showed very potent *S. aureus* UPP synthase (IC₅₀, 0.2 μM) and antimicrobial activities against *Enterococcus faecalis* (minimal inhibitory concentration, 8.0 μg ml⁻¹), *S. aureus* (4.0 μg ml⁻¹) and *S. pneumonia* (4.0 μg ml⁻¹). Unfortunately, the derivative **4a** showed decreased inhibitory activity in serum. In this study, we demonstrated that spirohexaline and viridicatumtoxin are potent UPP synthase inhibitors with antimicrobial activities against Gram-positive bacteria. The structures are classified into tetracycline-type antibiotics and do not resemble the substrate FPP. Thus, spirohexaline might not interact with the substrate-binding site on UPP synthase. Viridicatumtoxin was originally isolated as a mycotoxin;¹⁴ the single-dose LD₅₀ in mice was described as 122.4 mg kg⁻¹ in the initial report, but nonlethal oral administration up to 350 mg kg⁻¹ in mice was described in a later study,¹⁵ indicating that viridicatumtoxin showed very low cytotoxicity. Spirohexaline and viridicatumtoxin are of interest as new lead compounds of UPP synthase inhibitors. Further investigation on their biological properties will be reported shortly.

METHODS

Bacterial strains, plasmid, enzymes and chemicals

The *E. coli* strains used in this study were JM109 (Takara Bio Inc., Shiga, Japan) and BL21 (DE3) (Merck KGaA, Darmstadt, Germany). The strains were grown in Luria–Bertani (LB) medium (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) with aeration at 37 °C. Plasmid pET-42b was from

Table 1 Physicochemical properties of **1** and **2**

	Spirohexaline (1)	Viridicatumtoxin (2)
Appearance	Bright yellow pigment	Bright yellow pigment
Molecular formula	C ₃₁ H ₃₂ O ₁₀	C ₃₀ H ₃₁ NO ₁₀
HRFAB-MS (<i>m/z</i>)	563.1900 (M-H) ⁻	565.1962 (M +)
Found		
Calcd	563.1917	565.1948
UV(EtOH)λ _{max}	239(20 100), 281(35 800),	237(21 900), 285(32 900),
nm (ε)	423(8100)	424(8100)
[α] _D (c = 1.0 EtOH)	+ 13°	+ 12°
IRγ _{max} (cm ⁻¹)	3438, 2958, 2925, 1733, 1625, 1596	3550–3350, 2980, 1725, 1650, 1620, 1595

Table 2 ^1H and ^{13}C NMR data of **1** and **2**

Chemical shift for spirohexaline (1)			Chemical shift for viridicatumtoxin (2)		
Position	^{13}C	^1H	Position	^{13}C	^1H
1	190.2s		1	190.4s	
2	110.5s		2	99.5s	
3	194.4s		3	192.8s	
4	40.6t	2.82d (1H, $J=20$ Hz) 2.88d (1H, $J=20$ Hz)	4	40.3t	2.76dd (1H, $J=20$ Hz) 2.82dd (1H, $J=18$ Hz)
4a	71.1s		4a	71.5s	
5	71.5d	4.48br.s (1H)	5	71.6d	4.5br.s (1H)
5a	123.7s		5a	123.8s	
6	137.1s		6	137.0s	
6a	147.1s		6a	147.1s	
7	122.7s		7	122.6s	
8	160.7s		8	160.7s	
9	99.9d	6.65s (1H)	9	99.9d	6.65s (1H)
10	157.9s		10	157.9s	
10a	105.4s		10a	105.4s	
11	165.9s		11	165.9s	
11a	105.1s		11a	105.0s	
12	195.3s		12	195.2s	
12a	80.6s		12a	80.1s	
13	202.2s		13	172.7s	
14	41.1t	2.93d (1H, $J=17$ Hz) 3.44d (1H, $J=17$ Hz)	14	41.1t	2.93d (1H, $J=20$ Hz) 3.44d (1H, $J=20$ Hz)
15	60.1s		15	60.1s	
16	136.5s		16	136.6s	
17	121.4d	5.51br.s (1H)	17	121.3d	5.51br.s (1H)
18	22.8t	2.02 m (1H) 2.20 m (1H)	18	22.8t	2.02 m (1H) 2.20 m (1H)
19	33.8t	1.34dd (1H, $J=6,13$ Hz) 1.84ddd (1H, $J=6,12,12$ Hz)	19	33.8t	1.34dd (1H, $J=6,13$ Hz) 1.84ddd (1H, $J=3,13,13$ Hz)
20	38.5s		20	38.5s	
21	27.7q	2.73s (3H)	21	23.9q	1.53s (3H)
22	23.9q	1.53s (3H)	22	25.4q	0.47s (3H)
23	25.4q	0.48s (3H)	23	20.9q	0.91s (3H)
24	21.0q	0.91s (3H)	24	55.5q	3.87s (3H)
25	55.5q	3.87s (3H)	3-OH		17.9s (1H)
3-OH		18.1s (1H)	4a-OH		4.15s (1H)
4a-OH		4.10s (1H)	5-OH		3.04br.d (1H $J=10$ Hz)
5-OH		3.02d (1H, $J=12$ Hz)	10-OH		8.68br.s (1H)
10-OH		8.68s (1H)	11-OH		14.79br.s (1H)
11-OH		14.73s (1H)	12a-OH		5.28br.s (1H)
12a-OH		5.4br.s (1H)	13-NH ₂		5.92br.s (1H) 9.08br.s (1H)

Novagen (Abingdon, England). Restriction enzymes were from New England Biolabs (Beverly, MA, USA) or Takara Bio Inc. and used as specified by the manufacturers. Amplex red was from Invitrogen (Carlsbad, CA, USA). [$1\text{-}^{14}\text{C}$]IPP (1.48 GBq/mmol) was from PerkinElmer (Waltham, MA, USA). Pre-coated reversed-phase thin layer chromatography (TLC) plate, RP-18, was from GE Healthcare (Waukesha, WI, USA). All other chemicals were from Sigma (St Louis, MO, USA).

General experimental procedures

Fungal strain FKI-3368 was originally isolated from a soil sample collected on the Island of Hawaii, HI, USA. This strain was used for the production of **1** and **2**.

UV spectra were recorded on a spectrophotometer (8453 UV-visible spectrophotometer; Agilent Technologies, Santa Clara, CA, USA). IR spectra were recorded on a Fourier transform infrared spectrometer (FT-710, Horiba, Kyoto, Japan). Optical rotations were measured with a digital polarimeter

(DIP-1000, JASCO, Tokyo, Japan). FAB-MS spectra were recorded on a mass spectrometer (JMS-DX300, JEOL, Tokyo, Japan) and HRFAB-MS spectra were recorded on a mass spectrometer (JMS-AX505 HA, JEOL). The various NMR spectra were measured with a spectrometer (XL-400, Varian, Palo Alto, CA, USA).

Taxonomic studies

For determination of the morphological characteristics using the methodology of Pitt,¹⁶ the isolate was inoculated as 3-point cultures on CYA, malt extract agar and 25% glycerol nitrate agar, and grown for 7 days at 25 °C (also at 5 and 37 °C on CYA) in the dark. Color Harmony Manual 4th Edition (Container Corporation of America, Chicago, IL, USA) was used to determine color names and hue numbers.¹⁷

For the determination of micromorphological characteristics, microscope slides were prepared using malt extract agar. The slides were examined with a Vanox-S AH-2 microscope (Olympus, Tokyo, Japan).

Figure 3 HMBC correlation of spirohexaline.

Figure 4 Inhibition of UPP synthase by spirohexaline (■) and viridicatumtoxin (●).

Genomic DNA of the fungal strain FK1-3368 was isolated using the PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Amplifications of the ribosomal RNA gene (rDNA) ITS region, including the 5.8 S rDNA, the partial beta-tubulin gene region and the partial calmodulin gene region, were performed using primers ITS1 and ITS4¹⁸ for the ITS region, primers Bt2a and Bt2b¹⁹ for the beta-tubulin gene region and primers CF1 and CF4²⁰ for the calmodulin gene region. Amplifications were performed in a PCR Verity 96-well thermal cycler (Applied Biosystems) and the PCR products were purified using a QIAquick, PCR DNA Purification kit protocol (Qiagen Inc., Valencia, CA, USA). The PCR products were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing products were purified using DyeEX 2.0 Spin Kit (Qiagen), and samples were analyzed on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). Contigs were assembled using the forward and reverse sequences with the SeqMan and SeqBuilder programs from the Lasergene 9 package (DNASTar Inc., Madison, WI, USA). The DNA sequences of the strain FK1-3368 were deposited at the DNA Data Bank of Japan (DDBJ) with accession numbers AB455514 (ITS), AB667855 (β -tubulin) and AB667856 (calmodulin).

UPP synthase assays

UPP synthase reaction was measured by a coupling assay, a modification of the method of Vazquez *et al.*⁷, for inorganic phosphate determination. Reactions were performed in black 96-well plates from DS Pharma Biomedical Co. Ltd. (Osaka, Japan). UPP synthase was incubated at 37 °C with 5 mU of inorganic pyrophosphatase from *Saccharomyces cerevisiae* in a total reaction volume of 100 μ l containing 100 mM Tris-HCl (pH 7.5), 0.5 mM MgCl₂, 50 mM KCl,

3.5 μ M IPP, 0.5 μ M FPP and 0.005% (wt/vol) Triton X-100. The reaction was terminated after 30 min by the addition of 10 μ l of 0.5 M EDTA and quenched with 100 μ l of a cocktail containing 100 mM Tris-HCl (pH 7.4), 3 mM inosine, 0.2 mU ml⁻¹ PNP, 10 mU ml⁻¹ horseradish peroxidase, 4 mU ml⁻¹ XOD and 0.1 mM Amplex red. After another 30 min of incubation at room temperature, the plate was read at 530/590 nm. The amount of phosphate released from IPP was calculated from a standard curve prepared with KH₂PO₄.

Other analytical methods

Protein concentrations were determined using BCA protein assay kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). Minimal inhibitory concentrations were measured by the agar dilution method using Mueller Hinton Agar (BD, Franklin Lakes, NJ, USA). Freshly grown cells (or spores) of *S. aureus* ATCC6538p (methicillin-susceptible (MSSA)) and *S. aureus* K24 (MRSA) were inoculated and grown at 37 °C for bacteria.

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