ORIGINAL ARTICLE

Epi-trichosetin, a new undecaprenyl pyrophosphate synthase inhibitor, produced by *Fusarium oxysporum* FKI-4553

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A new compound, designated *epi*-trichosetin (1), was isolated along with the known compound trichosetin (2) from the culture broth of *Fusarium oxysporum* FKI-4553 by solvent extraction, silica gel column chromatography and reversed-phase HPLC. The structure of 1 was elucidated by comparing various spectral data with those of 2, revealing that 1 was a stereoisomer of 2. Compounds 1 and 2 inhibited the undecaprenyl pyrophosphate synthase activity of *Staphylococcus aureus* with IC₅₀ values of 83 and 30 μ M, respectively, and showed antimicrobial activity, particularly against Gram-positive bacteria, including methicillin-sensitive and -resistant *S. aureus*.

The Journal of Antibiotics (2013) 66, 549–554; doi:10.1038/ja.2013.44; published online 29 May 2013

Keywords: *epi*-trichosetin; *Fusarium oxysporum* FKI-4553; undecaprenyl pyrophosphate synthase inhibitor

INTRODUCTION

Antibiotic-resistant bacterial infections pose a major threat to public health. Methicillin-resistant *Staphylococcus aureus* (MRSA) is recognized as a major nosocomial pathogen that has also developed resistance to many other antibiotics, such as other β -lactams.¹ Moreover, MRSA resistant to the last-resort antibiotic, vancomycin, has been reported.² This suggests that *S. aureus* will acquire full resistance to vancomycin in the future. However, the past 30 years have seen only two new classes of antibiotics introduced into clinical use, represented by the natural product daptomycin³ and the synthetic oxazolidinone linezolid;⁴ therefore, there is a great need for the discovery and development of new anti-infective agents possessing novel mechanisms of action.

The bacterial cell wall is an attractive target for antibacterial agents because it is an essential, bacterial-specific structure that is absent in human cells.⁵ Undecaprenyl pyrophosphate (UPP) is a key lipid involved in the biosynthesis of peptidoglycan and other cell wall polysaccharide components such as lipopolysaccharides, enterobacterial common antigens, capsule polysaccharides and teichoic acids. UPP-linked saccharides are also used for *N*-linked protein glycosylation, which 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 externally located sites of polymerization. Thus, UPP synthase inhibitors that cause specific growth inhibition against bacteria, including MRSA or vancomycin-resistant *S. aureus*, are anticipated to be useful clinical

drugs because such inhibitors have yet to be used in the clinical or agricultural fields.

On the basis of this concept, screening for UPP synthase inhibitors of microbial origin was carried out, and spirohexaline and viridicatumtoxin were isolated and identified from a culture of *Penicillium brasilianum* FKI-3368.⁶ During the course of our continued screening program, a new compound, designated *epi*-trichosetin (1) (Figure 1), was isolated from the culture broth of *Fusarium oxysporum* FKI-4553 along with a known antibiotic, trichosetin (2).⁷ In this study, we describe the taxonomy of the producing fungus, and the fermentation, isolation, structure elucidation and activity of these trichosetins.

RESULTS

Taxonomy

Colonies on potato dextrose agar (PDA) were 72–74 mm in diameter after 7 days at 25 °C (Figure 2a), floccose, plane to subelevated, with white (1A1) to lilac (15B4) mycelium; the reverse was orange white (5A2) to dark violet (15F7). Colony margin was entire; exudate and soluble pigment were not produced. Colonies on synthetic low-nutrient agar (SNA) were more than 85 mm in diameter after 7 days at 25 °C, floccose, plane, with colorless to white (1A1) mycelium; exudate and soluble pigment were not produced. Colonies on PDA at 5 and 37 °C were not produced. Chlamydospores were formed frequently in hyphae and in conidia, mostly subglobose and single. Sclerotia were absent. Sporulation was generally rapid. Aerial conidia were of two types: (1) 3(–4)-septate conidia were curved

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Received 24 January 2013; revised 4 April 2013; accepted 12 April 2013; published online 29 May 2013

cylindrical to falcate, (20–) 25–52 $\times$ 3.8–6.0 (–7.0) μm^2 (Figure 2b); (2) 0(–1)-septate conidia were minute, obovate, short clavate to ellipsoidal, (4.3–)5.0–13.5 $\times$ 1.5–3.5 μm^2 (Figure 2c). Conidiogenesis of 3(–4)-septate conidia and 0(–1)-septate conidia was phialidic. From the above morphological characteristics, strain FKI-4553 was considered to belong to the genus *Fusarium*. In a BLAST search using blastn from the National Center for Biotechnology Information,⁸ FKI-4553 had 100% similarity with the internal transcribed spacer (ITS) sequences of *F. oxysporum* NRRL 22902 (GenBank accession number U34566). From these results of morphological characteristics and the homology searching using blastn, the producing strain FKI-4553 was identified with *F. oxysporum*.

Fermentation

A slant culture of *F. oxysporum* FKI-4553 grown on LCA medium (0.1% glycerol, 0.08% KH_2PO_4 , 0.02% K_2HPO_4 , 0.02% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02% KCl, 0.2% NaNO_3 , 0.02% yeast extract and 1.5% agar, adjusted to pH 6.0 before sterilization) was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of the seed medium (2.0% glucose, 0.5% polypeptone, 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2% yeast extract, 0.1% KH_2PO_4 and 0.1% agar, adjusted to pH 6.0 before sterilization). The flask was incubated on a rotary shaker (270 r.p.m.) for 3 days at 27 °C to obtain the seed culture. The production culture was initiated by transferring the seed culture (2 ml) into a 500-ml Erlenmeyer flask containing 100 ml of the production medium (3.0% soluble starch, 1.0% glycerol, 2.0% soybean meal, 0.3% dry yeast, 0.3% KCl, 0.2% CaCO_3 , 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05% KH_2PO_4 , adjusted to pH 6.0 before sterilization) and fermentation was carried out at 27 °C for 7 days. UPP synthase inhibitory activity was detected in the culture broth from day 3 after inoculation. The activity reached a maximal level on day 5 and kept almost the same levels at least on day 7.

Figure 1 Structures of *epi*-trichosetin (1) and trichosetin (2).

Isolation

The 7-day-old whole culture (3.8 l) was extracted with acetone (4.0 l). After the acetone extracts had been filtered and concentrated to remove acetone, the aqueous solution (3 l) was extracted twice with ethyl acetate (1.5 l). The extracts were dried over Na_2SO_4 and concentrated *in vacuo* to dryness to yield brown oily materials. The material (0.9 g) was dissolved in a small amount of CHCl_3 and applied to a silica gel column (i.d. 3.6 $\times$ 20 cm, silica gel 60, 70–230 mm; Merck KGaA, Darmstadt, Germany) previously equilibrated with CHCl_3 , and eluted stepwise with CHCl_3 -MeOH (200 ml each, 100:0, 9:1, 5:1, 2:1 and 0:100). The fractions of CHCl_3 -MeOH (5:1 and 2:1) were combined and concentrated *in vacuo* to dryness to give dark-brown material (145 mg). The material was finally purified by preparative HPLC (column, CAPCELL PAK C18, ϕ 20 $\times$ 250 mm; Shiseido Co Ltd, Tokyo, Japan; solvent, 62% CH_3CN containing 0.05% trifluoroacetic acid (TFA); detection, UV at 210 nm; flow rate, 8.0 ml min^{-1}). Under these conditions, 1 and 2 were eluted as peaks with retention times of 39.2 and 36.3 min, respectively. The fractions were concentrated *in vacuo* to dryness to give pure 1 (2.0 mg) and 2 (10.5 mg) as colorless materials.

Physicochemical properties

The physicochemical properties of 1 and 2 are summarized in Table 1. The UV spectra of these compounds showed similar absorption maxima at 250 and 288 nm or 249 and 289 nm, respectively, indicating very close structural relationship.

Structure elucidation

The molecular formula of *epi*-trichosetin (1) was determined to be $\text{C}_{21}\text{H}_{29}\text{NO}_4$ on the basis of HRESI-TOF-MS measurement (Table 1). The ^{13}C NMR spectrum (in CDCl_3) showed 21 resolved signals, which were classified into three methyl carbons, four methylene

Table 1 Physicochemical properties of *epi*-trichosetin (1) and trichosetin (2)

	1	2
Appearance	Colorless powder	Colorless powder
Molecular weight	359	359
Molecular formula	$\text{C}_{21}\text{H}_{29}\text{NO}_4$	$\text{C}_{21}\text{H}_{29}\text{NO}_4$
HRESI-TOF-MS (<i>m/z</i>)		
Calculated:	358.2018 (M-H) [–]	358.2018 (M-H) [–]
Found:	358.1903 (M-H) [–]	358.1900 (M-H) [–]
UV(MeOH) λ_{max} (nm) (ϵ)	249 (62 800), 289 (96 900)	250 (58 500), 288 (98 000)
$[\alpha]_D^{26}$ (<i>c</i> = 0.1, MeOH)	–233°	–413°
CD (MeOH) λ_{nm} (Mol. CD)	238 (0.17), 272 (–5.5)	230 (–4.6), 277 (–8.2)

Figure 2 *F. oxysporum* FKI-4553. (a) Photograph of colonies grown on PDA after 7 days. (b) Micrograph of 3–4 septate conidia grown on SNA. (c) Micrograph of 0–1 septate conidia grown on SNA. Scale bars: (b) 20 μm ; (c) 10 μm . A full color version of this figure is available at *The Journal of Antibiotics* journal online.

carbons, nine (four sp^2) methine carbons, three (two sp^2) quaternary carbons and two carbonyl carbons by analysis of DEPT spectra. The ^1H NMR spectrum (in CDCl_3) showed three methyl signals, five methylene signals and nine methine signals. The connectivity of proton and carbon atoms was established by the HMQC spectrum, as shown in Table 2. Analysis of the ^1H - ^1H COSY spectrum gave connectivity from C-3 to C-15 (Figure 3). Additional analysis of the HMBC experiments gave the following information. The cross peaks from H_3 -12 (δ 1.46) to C-1 (δ 200.8), C-2 (δ 49.3), C-3 (δ 45.2) and C-11 (δ 40.2) supported the partial structure I. The cross peaks from $5'$ -H (δ 3.89) to C-2' (δ 179.5) and C-3' (δ 100.3) and from $6'$ - H_2 (δ 3.79, 3.88) to C-4' (δ 191.1) supported the partial structure II. From

Figure 3 Key correlations in ^1H - ^1H COSY and HMBC spectra of **1**.

all these data and the results of searching the Chapman and Hall Dictionary of Natural Products database,⁹ the planar structure of **1** was elucidated to be the same as the known trichosetin (**2**).

The clear point of difference between **1** and **2** is the absolute value of optical rotation. The value of $[\alpha]_D^{25}$ of **1** was -233° in MeOH, whereas that of **2** was -413° , suggesting that **1** and **2** were stereoisomers. To elucidate the stereochemistry of **1**, ROESY experiments were carried out (Figure 4). Observation of the ROESY correlations between H-8ax (δ 1.50) and H-6ax (δ 1.84), between H-6ax and H_3 -12 (δ 1.46), between H_3 -12 and H-10ax (δ 1.05), and

Figure 4 Key ROESY correlations of **1**.

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

No.	1		2	
	δ_C^a	δ_H^b	δ_C^a	δ_H^b
1	200.8	—	200.6	—
2	49.3	—	49.0	—
3	45.2	3.32 (brd, $J=9.0$ Hz, 1H)	45.0	3.34 (brd, $J=9.0$ Hz, 1H)
4	126.8	5.38 (brs, **1H)	126.6	5.40 (brs, **1H)
5	130.2	5.39 (brs, **1H)	130.0	5.41 (brs, **1H)
6	38.7	1.84 (brt, $*J=12.0$ Hz, 1H)	38.5	1.85 (brt, $*J=12.0$ Hz, 1H)
7ax	42.5	0.88 (q, $*J=12.0$ Hz, 1H)	42.1	0.90 (q, $*J=12.0$ Hz, 1H)
7eq		1.80 (brd, $*J=12.0$ Hz, 1H)		1.85 (brd, $*J=12.0$ Hz, 1H)
8	33.7	1.50 (m, 1H)	33.4	1.52 (m, 1H)
9ax	35.9	1.09 (q, $J=12.0$ Hz, 1H)	35.6	1.11 (q, $J=12.0$ Hz, 1H)
9eq		1.75 (brd, $*J=12.0$ Hz, 1H)		1.76 (brd, $*J=12.0$ Hz, 1H)
10ax	28.6	1.05 (q, $*J=12.0$ Hz, 1H)	28.3	1.05 (q, $*J=12.0$ Hz, 1H)
10eq		1.96 (brd, $J=12.0$ Hz, 1H)		1.96 (brd, $J=12.0$ Hz, 1H)
11	40.2	1.67 (brt, $*J=12.0$ Hz, 1H)	40.0	1.68 (brt, $*J=12.0$ Hz, 1H)
12	14.1	1.46 (s, 3H)	13.9	1.46 (s, 3H)
13	131.0	5.16 (dd, $J=15.0, 9.0$ Hz, 1H)	130.8	5.20 (dd, $J=15.0, 9.0$ Hz, 1H)
14	127.2	5.24 (dq, $J=15.0, 6.5$ Hz, 1H)	127.2	5.27 (dq, $J=15.0, 6.5$ Hz, 1H)
15	18.1	1.52 (d, $J=6.5$ Hz, 3H)	17.9	1.55 (d, $J=6.5$ Hz, 3H)
16	22.7	0.91 (d, $J=6.5$ Hz, 3H)	22.4	0.92 (d, $J=6.5$ Hz, 3H)
1'	—	5.91 (brs, 1H)	—	6.44 (brs, 1H)
2'	179.5	—	179.1	—
3'	100.3	—	99.9	—
4'	191.1	—	190.6	—
5'	61.8	3.89 (dd, $J=5.0, 3.5$ Hz, 1H)	62.2	3.89 (dd, $J=5.0, 3.5$ Hz, 1H)
6'	63.2	3.79 (dd, $J=12.0, 3.5$ Hz, 1H)	62.9	3.82 (dd, $J=12.0, 3.5$ Hz, 1H)
		3.88 (dd, $J=12.0, 3.5$ Hz, 1H)		3.90 (dd, $J=12.0, 3.5$ Hz, 1H)

*Partially obscured; **overlapping.

^aChemical shifts are shown with reference to CDCl_3 as 77.0 p.p.m.

^bChemical shifts are shown with reference to CDCl_3 as 7.26 p.p.m.

Figure 5 Inhibition of UPPS by **1** (■) and **2** (●). Prenylsynthase activity was determined to measure the amount of free phosphate by an enzyme-coupling fluorescent method, as described in the General experimental procedure. Error bars: $\pm$ s.d. ($n=4$).

Table 3 Antimicrobial activity of **1** and **2**

Test organism	Inhibition zone (mm) ^a	
	1	2
Gram-positive bacteria		
<i>Staphylococcus aureus</i> ATCC 6538P (MSSA)	18	15
<i>S. aureus</i> K24 (MRSA)	16	14
<i>Bacillus subtilis</i> PCI 219	18	16
<i>Micrococcus luteus</i> PCI 9341	17	15
<i>Mycobacterium smegmatis</i> ATCC 607	9	8
Gram-negative bacteria		
<i>Escherichia coli</i> NIHJ	10	10
<i>Pseudomonas aeruginosa</i> IFO 3080	—	—
<i>Xanthomonas campestris</i> KB 88	9	7
<i>Bacteroides fragilis</i> ATCC 23745	11	11
Mycoplasma		
<i>Acholeplasma laidlawii</i> KB 174	12	9
Molds		
<i>Pyricularia oryzae</i> KF 180	14	11
<i>Aspergillus niger</i> ATCC 9642	—	—
<i>Mucor racemosus</i> IFO 4581	7	7
Yeasts		
<i>Candida albicans</i> ATCC 64548	—	—
<i>Saccharomyces cerevisiae</i> ATCC 9763	—	—

Abbreviations: MRSA, methicillin-resistant *Staphylococcus aureus*.
^aConcentration of the sample; 10 μ g per 6 mm paper disk.

between H-8ax and H-10ax in **1** suggested that all these protons were located on the same side of the molecule. Similarly, H-7ax (δ 0.88), H-9ax (δ 1.09), H-11 (δ 1.67) and H-13 (δ 5.16) were located on the other side of the molecule. From these results, the absolute configuration of **1** was determined as 2*S*, 3*S*, 6*S*, 8*R*, 11*R* (Figure 4). The structural difference between **1** and **2** was derived from the stereochemistry at C-5'. In fact, the chemical shifts at 1'-NH were distinct (δ 5.91 for **1** and δ 6.44 for **2**), indicating that C-5' in **1** should be *R* because **2** was reported to have *S* stereochemistry.⁷ Taken together, the absolute configuration of **1** was elucidated to be 2*S*, 3*S*, 6*S*, 8*R*, 11*R*, 5'*R*. Thus, **1** was the epimer of **2** at C-5'.

Biological properties

UPP synthase activity. UPP synthase activity was measured by the enzyme-coupled fluorescent method.¹⁰ Compounds **1** and **2** inhibited UPP synthase activity, with IC₅₀ values of 83 and 30 μ M, respectively (Figure 5).

Antimicrobial activity. As **1** and **2** showed moderate inhibition of UPP synthase, it was interesting to see whether they showed antimicrobial activity. Table 3 summarizes the results by the paper disk method. Compounds **1** and **2** showed broad antibacterial activity, in particular, potent activity against Gram-positive bacteria, including methicillin-sensitive *S. aureus* (ATCC 6538P, MSSA) and -resistant *S. aureus* (K24, MRSA). Compound **1** appeared more potent than **2** by this method.

DISCUSSION

F. oxysporum FKI-4553 produced two structurally related compounds **1** and **2** at an approximate ratio of 1:5. The structure of **1** was elucidated by comparing its spectral data with those of **2**. Careful comparison enabled us to conclude that **1** was the epimer of **2** at C-5'. Several structurally related compounds, such as equisetin (**3**),¹¹ paecilisetin (**4**),¹² and phomasetin (**5**),¹³ were isolated from fungal strains belonging to the genera *Fusarium*, *Phoma* and *Paecilomyces*, respectively (Figure 6). All compounds having a tetramic acid moiety are known to show a variety of biological activities, such as antimicrobial activity,¹⁴ phytotoxic activity¹⁵ and inhibitory activity of HIV integrase.^{13,16,17} From the present study, it is plausible that antimicrobial activity of **1** and **2** (Table 3) was caused by the inhibition of UPP synthase. However, these compounds also inhibited fungal growth. It might be that a tetramic acid has potential to bind to various proteins, including UPP synthase-related fungal enzymes such as dehydrololichyl diphosphate synthase,¹⁸ leading to a variety of biological activities.

Figure 6 Structures of equisetin (**3**), paecilisetin (**4**) and phomasetin (**5**).

At first, **1** was thought to be an artifact from **2**, because Phillips *et al.*¹⁹ reported the possibility of epimerization of equisetin by pyridine treatment, but pyridine was not used during the isolation process from the culture broth. From the study of trichosetin biosynthesis,²⁰ **2** was produced via the polyketide pathway, and C-5' was derived from serine.²¹ Therefore, the polyketide synthase for **2** might recognize D/L-serine or possess epimerase activity; however, we cannot rule out that **1** is an artifact of **2**. This point will be clarified by the functional analysis of the biosynthetic genes of natural products involved in the tetramic acid moiety.

MATERIALS AND METHODS

Bacterial strains, plasmid, enzymes and chemicals

F. oxysporum FKI-4553 was used for the production of **1** and **2**.

The *Escherichia coli* strains used for the production of the recombinant UPP synthase were JM109 (Takara Bio Inc, Shiga, Japan) and BL21 (DE3) (Merck KGaA). The strains were grown in Luria–Bertani medium (Becton Dickinson and Company, Franklin Lakes, NJ, USA), with aeration at 37 °C. Microorganisms used for measurement of antimicrobial activity were as follows: *Bacillus subtilis* PCI 219, *S. aureus* ATCC 6538P, *S. aureus* K24 (MRSA), *Micrococcus luteus* PCI 9341, *Mycobacterium smegmatis* ATCC 607, *E. coli* NIHJ, *Pseudomonas aeruginosa* IFO 3080, *Xanthomonas campestris* KB 88, *Bacteroides fragilis* ATCC 23745, *Acholeplasma laidlawii* KB 174, *Pyricularia oryzae* KF 180, *Aspergillus niger* ATCC 9642, *Mucor racemosus* IFO 4581, *Candida albicans* ATCC 64548 and *Saccharomyces cerevisiae* ATCC 9763.

Plasmid pET-42b(+) was from Novagen (Abingdon, England). Restriction enzymes were from New England Biolabs (Beverly, MA, USA) or Takara Bio Inc, and were used as specified by the manufacturers. Amplex red was from Invitrogen (Carlsbad, CA, USA). [1-¹⁴C] IPP (1.48 GBq mmol⁻¹) was from PerkinElmer (Waltham, MA, USA). The precoated reversed-phase TLC plate, RP-18, was from GE Healthcare (Buckinghamshire, UK). All other chemicals were from Sigma (St Louis, MO, USA).

General experimental procedures

SSC-ODS-7515-12 (Senshu Scientific Co, Tokyo, Japan) was used for the reversed-phase (C18) HPLC. HPLC was carried out using the L-6200 system (Hitachi Ltd, Tokyo, Japan). UV spectra were recorded on a spectrophotometer (8453 UV–Visible spectrophotometer; Agilent Technologies Inc, Santa Clara, CA, USA). Optical rotations were measured with a digital polarimeter (DIP-1000; JASCO, Tokyo, Japan). HR-ESI-TOF-mass spectra were recorded on a mass spectrometer (JMS-T100LP; JEOL, Tokyo, Japan). Various NMR spectra were measured with a spectrometer (XL-400; Agilent Technologies, Inc., Loveland, CO, USA).

Taxonomic studies of the producing organism

Fungal strain FKI-4553 was isolated from soil collected in Yamaguchi City, Yamaguchi Pref., Japan. For determination of the morphological characteristics, the isolate was inoculated as a one-point culture on PDA and SNA, and kept at 25 °C for 7 days (also at 5 and 37 °C on PDA) in the dark. The *Methuen Handbook of Color* was used to determine color names and hue numbers.²² For the determination of micro-morphological characteristics, microscope slides were prepared using SNA. The slides were examined with a Vanox-S AH-2 microscope (Olympus, Tokyo, Japan).

Genomic DNA of fungal strain FKI-4553 was isolated using PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions. Amplification of the ribosomal RNA gene (rDNA) ITS region including the 5.8S rDNA was performed using primers ITS1 and ITS4.²³ 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 (Qiagen Inc, Valencia, CA, USA), following the manufacturer's instructions. The PCR products were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing products were purified using a BigDye XTerminator (Applied Biosystems), 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 ITS sequences of the strain FKI-4553 were deposited in DNA Data Bank of Japan with the accession number AB685181.

UPP synthase assay

UPP synthase activity was measured as described elsewhere.^{6,10} In brief, 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 µl containing 100 mM Tris-HCl (pH 7.5), 0.5 mM MgCl₂, 50 mM KCl, 3.5 µM isopentenyl pyrophosphate, 0.5 µM farnesyl pyrophosphate and 0.005% (wt/vol) Triton X-100. The reaction was terminated after 30 min by the addition of 10 µl of 0.5 M EDTA and quenched with 100 µl of a cocktail containing 100 mM Tris-HCl (pH 7.4), 3 mM inosine, 0.2 mU ml⁻¹ PNP, 10 mU ml⁻¹ HRP, 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.

Antimicrobial assays

Antimicrobial activity against 15 species of microorganisms was measured by the paper disk method.²⁴ Media for microorganisms were as follows: nutrient agar (Sanko Junyaku Co, Ltd, Tokyo, Japan) for the bacteria, except *M. smegmatis*; a medium composed of 0.5% peptone, 0.5% meat extract, 0.3% NaCl, 1.0% glucose and 0.8% agar for *M. smegmatis*; a medium composed of 1.0% glucose, 0.5% yeast extract and 0.8% agar for fungi and yeasts. Bacteria, except *X. oryzae*, were incubated at 37 °C for 24 h. Yeasts, *A. niger* and *X. oryzae*, were incubated at 27 °C for 24 h. *P. oryzae* and *M. racemosus* were incubated at 27 °C for 48 h. *B. fragilis* was incubated under anaerobic conditions. A paper disk (6 mm) containing a sample (10 µg) was placed on an agar plate. Antimicrobial activity of a sample was expressed as the diameter (mm) of the inhibitory zone.

ACKNOWLEDGEMENTS

We wish to thank Dr K. Nagai and Ms N. Sato (Kitasato University) for measurements of mass spectra and NMR, respectively.

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