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BRIEF COMMUNICATION

Pestiocandin, a new papulacandin class antibiotic isolated from *Pestalotiopsis humus*

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Abstract

Secondary metabolites of microorganisms have proven to be an excellent source of drugs. We isolated a new antibiotic, named pestiocandin (**1**), from a culture broth of a filamentous fungus, *Pestalotiopsis humus* FKI-7473, using a multidrug-sensitive budding yeast, *S. cerevisiae* 12geneΔ0HSR-iERG6. The structure of **1** was elucidated by various NMR studies. All geometric isomerisms of **1** were shown to be the *E*-form and two pyranose units of **1** were found to be glucose and galactose types. Compound **1** showed weak growth inhibition against Gram-positive and Gram-negative bacteria, yeasts and a filamentous fungus. It displayed more potent growth inhibition against multidrug-sensitive yeasts than wild-type yeasts.

Natural products are an extremely useful source of chemicals for development into drugs, pesticides and reagents because they generally demonstrate a diversity of unique biological activities or chemical structures [1]. Secondary metabolites of microorganisms, such as penicillin, streptomycin and amphotericin B, have been used as highly successful medications in human health. However, it is not easy to discover potentially useful drug candidates from natural products. Thus, a new screening system is often required to promote exploratory research to unearth novel chemicals.

We have screened microbial culture broths to identify secondary metabolites that specifically inhibit the drug-sensitive recombinant *Saccharomyces cerevisiae*. The budding yeast, *S. cerevisiae*, is a model organism of eukaryotes and it is often used in screening and target identification of bioactive compounds from natural product sources [2, 3]. However, the high level of drug resistance already reported in *S. cerevisiae* has complicated the screening process. Consequently, a multidrug-sensitive budding yeast, *S. cerevisiae* 12geneΔ0HSR-iERG6, has been produced which is a very useful tool for antifungal screening because the resistant strain is devoid of a drug efflux system, and its expression of the gene related to the permeability barrier is a down-regulated system in the presence of glucose [4, 5]. Using the *S. cerevisiae* 12geneΔ0HSR-iERG6 screening system, we isolated a new compound, named pestynol [6], from a culture broth of the filamentous fungus, *Pestalotiopsis humus* FKI-7473. Here, we report another new antibiotic, named pestiocandin (**1**), isolated from a culture broth of the same fungus.

One loopful of strain FKI-7473, grown on an LcA slant (0.1% glycerol, 0.08% KH_2PO_4, 0.02% K_2HPO_4, 0.02% $MgSO_4{\cdot}7H_2O$, 0.02% KCl, 0.2% $NaNO_3$ and 1.5% agar, pH 6.0), was inoculated into a 500-mL Erlenmeyer flask containing 100 mL of a seed medium (2% glucose, 0.2% yeast extract, 0.05% $MgSO_4{\cdot}7H_2O$, 0.5% polypepton (FUJIFILM Wako Pure Chemical Co., Osaka, Japan), 0.1% KH_2PO_4 and 0.1% agar, pH 6.0) and incubated on a rotary shaker at 27 °C for 4 days. Twenty-five millilitres of the seed culture was inoculated into each of 20 Ulpack 47 (culture bags) (HOKKEN Co. Ltd., Tochigi, Japan) containing a

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production medium (500 g of water-sodden rice). Static fermentation was carried out at 27 °C for 14 days. The stationary culture was extracted with acetone (18 L). After centrifugation, the supernatant was collected and concentrated in vacuo to remove acetone. The aqueous solution (4 L) was extracted three times with an equal volume of EtOAc. The organic layer was concentrated to dryness to afford a crude extract (50 g). The extract was chromatographed on a silica gel (Silica gel 60 (0.063–0.200 mm), Merck KGaA, Darmstadt, Germany) column and eluted stepwise with a mixture of $CHCl_3$–MeOH (100:0, 100:1, 100:5, 100:10, 1:1 and 0:100). The 1:1 fraction (5.7 g) was applied to an ODS silica gel (YMC*GEL ODS-A, YMC Co., Ltd., Kyoto, Japan) column (60 i.d. × 140 mm) and eluted stepwise with a mixture of MeOH–H_2O (20:80, 30:70, 50:50, 70:30, 80:20, 90:10 and 100:0). The 80:20 fraction was concentrated in vacuo to remove solvents.

The dried material (197 mg) was dissolved in a small amount of MeOH and applied to preparative HPLC (column, Pegasil ODS SP100: size, 20 i.d. × 250 mm; Senshu Scientific Co. Ltd., Tokyo, Japan) with an isocratic solvent system of 45% CH_3CN at a flow rate of 10 mL/min. The eluted peak, from 80 to 84 min, was collected to give 21.5 mg of **1** (Fig. 1a) as a yellow oil. It was soluble in MeOH, acetonitrile, $CHCl_3$ and DMSO but not in H_2O. The compound showed $[\alpha]_D^{23} + 21$ ($c = 0.1$, CH_3OH), IR (ATR) ν_{max} 3362, 2925, 2850, 2359, 2335, 1716, 1699, 1653, 1558, 1541, 1457 1264, 1150, 1043 cm^{-1} and UV (MeOH) λ_{max} nm(ε) 206 (65,600), 223 (43,200), 263 (30,500). The molecular formula of **1** was identified as $C_{43}H_{62}O_{16}Na$ (found *m/z* 857.3962 $[M+Na]^+$, calculated *m/z* 857.3936 $[M+Na]^+$) by HR-ESIMS. All connections for ^1H and ^{13}C in **1** were revealed by an HSQC study. The NMR data showed the presence of two methyl, 12 methylene, 11 sp^3 methine, 12 sp^2 methine, four fully substituted sp^2 and two carbonyl carbons (Table 1). ^1H–^1H correlations in COSY from H-1 (δ_H 4.80) to H-6 (δ_H 3.89 and 4.04) showed the existence of a sugar moiety. The glycoside was elucidated to be the glucose type through analysis of the coupling patterns of proton signals, ROESY correlations (Fig. S7) and HOHAHA spectra (Fig. S8). Furthermore, the $J_{H1,2}$ value ($J = 10.0$ Hz) indicated that it is a β-glucoside. The HMBC correlations from H-9 (δ_H 6.28) to C-7 (δ_C 114.4), C-8 (δ_C 158.7), C-10 (δ_C 159.3) and C-11 (δ_C 109.4), from H-11 (δ_H 6.41) to C-7, C-9 (δ_C 104.5), C-10, C-12 (δ_C 143.1) and C-13 (δ_C 63.7) and from H-13 (δ_H 4.55 and 4.64) to C-7, C-11 and C-12 proved the existence of a 3,5-dihydroxybenzyl alcohol unit. HMBC correlations from H-1 to C-7, C-8 and C-12 showed that this unit was combined with the glucose unit by a *C*-glycosidic bond. ^1H–^1H COSY between H-1′ (δ_H 4.35) and H-2′ (δ_H 3.48), H-3′ (δ_H 3.46) and H-4′ (δ_H 3.75) and H-5′ (δ_H 3.67) and H-6′ (δ_H 4.13 and 4.20) and HMBC correlations from H-1′ to C-3′ (δ_C 74.7), from H-2′ to C-1′ (δ_C 105.4) and C-3′, from H-3′ to C-1′ and C-2′ (δ_C 72.6), from H-4′ to C-2′ and C-3′, from H-5′ to C-1′, C-3′, C-4′ (δ_C 70.2) and C-6′ (δ_C 64.5) and from H-6′ to C-4′ and C-5′ (δ_C 73.9) indicated the presence of another sugar moiety containing an anomeric proton. The glycoside was elucidated to be the galactose type through analysis of the coupling patterns of proton signals, ROESY (Fig. S7) and HOHAHA spectra (Fig. S9). Moreover, the $J_{H1',2'}$ value ($J = 7.1$ Hz) showed that it is a β-galactoside. HMBC correlations from H-4 (δ_H 3.97) to C-1′ and from H-1′ to C-4 indicated that the galactose connected to the glucose unit through an *O*-glycosidic bond. Connections from C-2′′ (δ_C 121.6) to C-12′′ (δ_C 18.2) were elucidated by ^1H–^1H COSY. In addition, the presence of the oxygenated hydrocarbon unit was proved by HOHAHA spectra observed from H-12′′ (δ_H 1.74) to H-7′′ (δ_H 4.14) (Fig. S10) and from H-3′′ (δ_H 7.25) to H-7′′ (Fig. S11) and decoupling of proton signals of H-6′′ (δ_H 2.38), H-8′′ (δ_H 5.53), H-11′′ (δ_H 5.69) and H-3′′ (Figs. S12–14). HMBC correlations from H-2′′ (δ_H 5.87), H-3′′ (δ_H 7.25) and H-3 (δ_H 5.18) to C-1′′ (δ_C 168.9) indicated that this unit was connected to the glucose unit through the carbonyl carbon of C-1′′. The remaining 12 carbons were suggested to be an

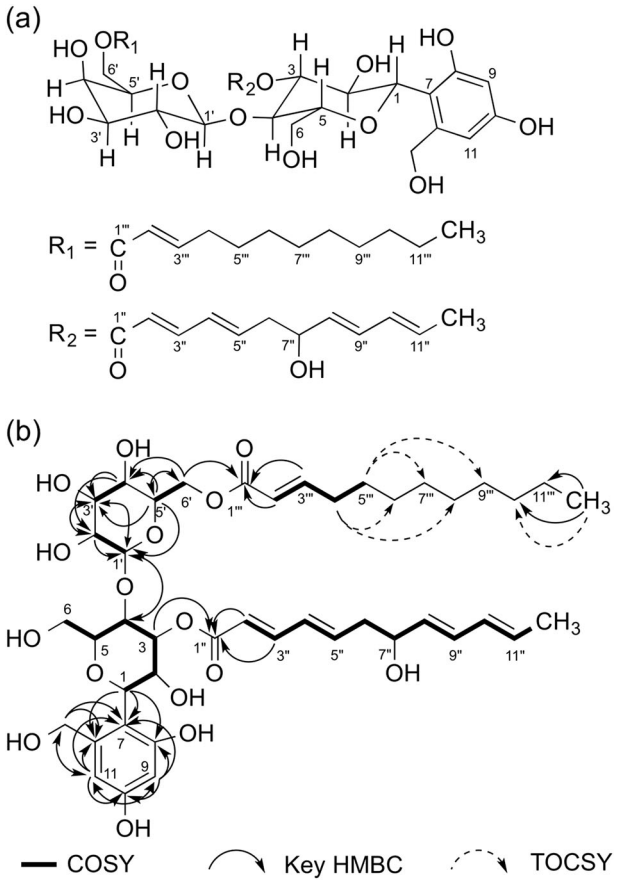

Fig. 1 **a** Structure of pestiocandin (**1**). **b** Key correlations of ^1H–^1H COSY and HMBC in pestiocandin (**1**)

Table 1 NMR spectroscopic data for pestiocandin (**1**) in CD_3OD

Position	δ_C	Type of carbon	δ_H (int., mult., J in Hz)
1	78.6	CH	4.80 (1H, d, $J = 10.0$ Hz)
2	71.9	CH	3.99 (1H, dd, $J = 10.0$, 9.4 Hz)
3	79.0	CH	5.18 (1H, dd, $J = 9.4$, 9.4 Hz)
4	77.4	CH	3.97 (1H, dd, $J = 10.0$, 9.4 Hz)
5	81.3	CH	3.63 (1H, ddd, $J = 10.0$, 3.0, 2.0 Hz)
6	61.0	CH_2	3.89 (1H, dd, $J = 12.5$, 2.0 Hz)
			4.04 (1H, dd, $J = 12.5$, 3.0 Hz)
7	114.4	C	
8	158.7	C	
9	104.5	CH	6.28 (1H, d, $J = 2.4$ Hz)
10	159.3	C	
11	109.4	CH	6.41 (1H, d, $J = 2.4$ Hz)
12	143.1	C	
13	63.7	CH_2	4.55 (1H, d, $J = 12.4$ Hz)
			4.64 (1H, d, $J = 12.4$ Hz)
1′	105.4	CH	4.35 (1H, d, $J = 7.1$ Hz)
2′	72.6	CH	3.48 (1H, dd, $J = 10.0$, 7.1 Hz)
3′	74.7	CH	3.46 (1H, dd, $J = 10.0$, 1.0 Hz)
4′	70.2	CH	3.75 (1H, d, $J = 1.0$ Hz)
5′	73.9	CH	3.67 (1H, dd, $J = 7.0$, 6.0 Hz)
6′	64.5	CH_2	4.13 (1H, dd, $J = 11.2$, 7.0 Hz)
			4.20 (1H, dd, $J = 11.2$, 6.0 Hz)
1″	168.9	C	
2″	121.6	CH	5.87 (1 H, d, $J = 15.5$ Hz)
3″	146.1	CH	7.25 (1 H, dd, $J = 15.5$, 10.1 Hz)
4″	131.9	CH	6.27 (1 H, dd, $J = 16.0$, 10.1 Hz)
5″	141.3	CH	6.13 (1 H, dt, $J = 16.0$, 7.2 Hz)
6″	42.2	CH_2	2.38 (2 H, m)
7″	72.6	CH	4.14 (1 H, dt, $J = 7.1$, 5.6 Hz)
8″	133.9	CH	5.53 (1 H, dd, $J = 14.9$, 7.1 Hz)
9″	132.0	CH	6.17 (1 H, dd, $J = 14.9$, 10.6 Hz)
10″	132.3	CH	6.04 (1 H, dd, $J = 14.9$, 10.6 Hz)
11″	130.4	CH	5.69 (1 H, dq, $J = 14.9$, 6.9 Hz)
12″	18.2	CH_3	1.74 (3 H, d, $J = 6.9$ Hz)
1‴	168.0	C	
2‴	121.9	CH	5.91 (1 H, d, $J = 15.6$ Hz)
3‴	151.9	CH	7.05 (1 H, dt, $J = 15.6$, 6.7 Hz)
4‴	33.3	CH_2	2.25 (2 H, dt, $J = 7.5$, 6.7 Hz)
5‴	29.2	CH_2	1.49 (2 H, m)
6‴	30.4	CH_2	1.34[a] (2 H, m)
7‴	30.5	CH_2	1.32[a] (2 H, m)
8‴	30.7	CH_2	1.30[a] (2 H, m)
9‴	30.4	CH_2	1.36[a] (2 H, m)
10‴	33.0	CH_2	1.29[a] (2 H, m)
11‴	23.7	CH_2	1.30[a] (2 H, m)
12‴	14.5	CH_3	0.90 (3 H, t, $J = 6.8$ Hz)

Data were collected at 400 MHz for ^1H and 100 MHz for ^{13}C

[a]Overlapped

unsaturated fatty acid by 1D and 2D NMR data including HSQC-TOCSY (Fig. S15). The alignment from C-2‴ (δ_C 121.9) to C-5‴ (δ_C 29.2) was elucidated by ^1H–^1H COSY between H-2‴ (δ_H 5.91) and H-3‴ (δ_H 7.05), H-3‴ and H-4‴ (δ_H 2.25) and H-4‴ and H-5‴ (δ_H 1.49), and the alignment from C-10‴ (δ_C 33.0) to C-12‴ (δ_C 14.5) was elucidated by HMBC correlations from H-12‴ (δ_H 0.90) to C-10‴ and C-11‴ (δ_C 23.7). The remaining four carbons were assigned as methylene carbons by HSQC, and their alignment was shown by HSQC-TOCSY correlations from H-4‴ to C-6‴ (δ_C 30.4) and C-8‴ (δ_C 30.7), from H-5‴ to C-7‴ (δ_C 30.5) and C-9‴ (δ_C 30.4) and from H-12‴ to C-10‴ (δ_C 33.0). HMBC correlations from H-2‴, H-3‴ and H-6′ to C-1‴ (δ_C 168.0) indicated that it was connected to the galactose unit through the carbonyl carbon of C-1‴. Thus, the planar structure of **1** was concluded to be as shown in Fig. 1b. All geometric isomerisms of **1** were elucidated to be the *E*-form by large coupling constants of $J_{H2'',3''}$, $J_{H4'',5''}$, $J_{H8'',9''}$, $J_{H10'',11''}$ and $J_{H2''',3'''}$. Compound **1** belongs to the papulacandin class of antibiotics [7, 8] and, to our knowledge, this represents the first report of the isolation of **1** from *Pestalotiopsis* sp.

There are two types of connection between glucose unit and the benzyl alcohol in papulacandin class antibiotics. The papulacandin type has a spirocyclic moiety, whereas the chaetiacandin type, including **1**, does not [9, 10]. Two types of galactose units have been reported for chaetiacandin-type antibiotics, a β-galactofuranoside type (like furanocandin [10]) and β-galactopyranoside type (like the fusacandins A and B [11]). Compound **1** contains β-galactopyranoside as shown above and is confirmed by its carbon chemical shifts [12].

Minimum inhibitory concentration (MIC) values of **1** were evaluated against Gram-positive and Gram-negative bacteria, yeasts and a filamentous fungus by a liquid dilution method using 96-well microplates. Compound **1** showed moderate or weak growth inhibition against all Gram-positive bacteria tested as well as inhibition in some Gram-negative bacteria, yeasts and a filamentous fungus (Table 2).

In yeast, our tests showed that 12 genes related to drug sensitivity were disrupted in *S. cerevisiae* 12geneΔ0HSR-iERG, while four genes related to drug sensitivity were disrupted in *S. cerevisiae* BY25929. Compound **1** showed moderate growth inhibition against *S. cerevisiae* 12geneΔ0HSR-iERG6, and weak growth inhibition against *S. cerevisiae* BY25929. Conversely, it did not inhibit growth in wild-type yeasts such as *S. cerevisiae* BY4741. Papulacandin class antibiotics, such as the papulacandins [13], L-687,781 [14], fusacandins A and B [15] and F-10748 C_1 and C_2 [16], have been reported to inhibit fungal β(1,3)-D-glucan synthase. However, **1** did not show potent antifungal activity.

Table 2 MIC values of pestiocandin (**1**)

Organism	MIC[a] values (μg/mL)		
	Pestiocandin	MEPM[b]	AMPH-B[c]
Gram-positive bacteria			
Staphylococcus aureus KB210 (ATCC6538p)	128	0.05	N.T.
Bacillus subtilis KB211 (ATCC6633)	8	1.6	N.T.
Micrococcus luteus KB212 (ATCC9341)	128	0.05	N.T.
Mycobacterium smegmatis KB42 (ATCC607)	256	2	N.T.
Gram-negative bacteria			
Escherichia coli KB213 (NIHJ)	> 256	0.03	N.T.
Klebsiella pneumoniae KB214 (ATCC10031)	> 256	0.03	N.T.
Pseudomonas aeruginosa KB115 (IFO3080)	> 256	0.03	N.T.
Xanthomonas oryzae pv. *oryzae* KB88	16	0.03	N.T.
Proteus vulgaris KB127 (NBRC 3167)	64	0.05	N.T.
Yeast			
Candida albicans KF1 (ATCC 64548)	> 256	N.T.	0.5
Saccharomyces cerevisiae KF237 (ATCC9763)	> 256	N.T.	8
Saccharomyces cerevisiae BY4741	> 256	N.T.	16
Saccharomyces cerevisiae BY25929	128	N.T.	1
Saccharomyces cerevisiae 12geneΔ0HSR-iERG6	64	N.T.	0.5
Filamentous fungus			
Mucor racemosus KF223 (IFO4581)	256	N.T.	0.06

N.T. not tested

All tested bacteria (McFarland No. 0.5 solution diluted 200-fold/100 μL/well in a 96-well plate) were cultured in MHB. All tested yeast (1.0×10^4 cells/100 μL/well in a 96-well plate) were cultured in YPD medium. *M. racemosus* KF223 (2.5×10^2 CFU/100 μL/well in a 96-well plate) was cultured in YPD medium.

[a]MIC (μg/mL), minimum inhibitory concentration

[b]MEPM, meropenem trihydrate

[c]AMPH-B, amphotericin B

In conclusion, a new papulacandin class antibiotic was isolated from the fungus, *Pestalotiopsis humus* FKI-7473. Our research has also confirmed that multidrug-sensitive *S. cerevisiae* organisms have become useful tools to facilitate isolation and identification of potentially promising compounds for development into much-needed new antibiotics.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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