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Rhodopeptins (Mer-N1033)†, Novel Cyclic Tetrapeptides with Antifungal Activity from *Rhodococcus* sp.

I. Taxonomy, Fermentation, Isolation, Physico-chemical Properties and Biological Activities

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Five novel cyclic tetrapeptides, named rhodopeptin C1, C2, C3, C4 and B5, were isolated from a strain named *Rhodococcus* sp. Mer-N1033. They are a novel type of cyclic tetrapeptide composed of a β-amino acid and three usual α-amino acids. Rhodopeptins show high *in vitro* antifungal activity against *Candida albicans* and *Cryptococcus neoformans*, whereas they show no activity against bacteria.

In the course of our screening for antifungal substances from microbial origins, we found that a strain of *Rhodococcus* sp. produced a series of cyclic tetrapeptides that strongly inhibited the growth of *Candida albicans*. Five of them, namely rhodopeptin C1, C2, C3, C4 and B5, were isolated from the bacterial pellet, which forms cell cake, of the strain. They are novel cyclic tetrapeptides, Fig. 1, composed of a lipophilic β-amino acid and three usual α-amino acids.[1] In this paper we describe the taxonomy and fermentation of the producing microorganism, isolation, physico-chemical properties, and some biological activities of the antibiotics.

Results

Taxonomy of the Producing Organism

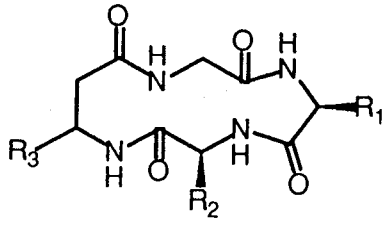
The producing organism, strain Mer-N1033, was isolated from a soil sample collected at Mt. Hayachine, Iwate Prefecture, Japan. Most of the cultural and physiological characteristics of the strain were examined using the methods described in BERGEY's Manual of Systematic Bacteriology.[2] The cells were spherical or ellipsoidal in stationary phase, about 1.0~1.2×1.2~1.5 μm in diameter. After inoculation to fresh medium, cells germinated into rods, 3.0~5.0 μm or longer. Fragmentation of rods succeeded, but no branched hyphae were observed. Aerial mycelium was never formed. Growth on Brain Heart Infusion Agar (Difco Laboratories) was medium, and the color of the colonies was pale yellow.

The detection of the isomers of diaminopimelic acid and whole cell sugars was performed by the methods of HASEGAWA *et al.*[3] The preparation and analysis of mycolic acid were studied according to the method of YANO *et al.*,[4] comparing with that of *Rhodococcus equi* ATCC 6939, which was purchased from American Type Culture Collection. The cell wall of the strain Mer-N1033 contained *meso*-diaminopimelic acid, arabinose, and galactose. Mycolic acid was also detected. Production of catalase was positive.

From its obvious rod-coccus growth cycle and its cell wall composition, the strain Mer-N1033 was classified as the genus *Rhodococcus* and was named *Rhodococcus* sp.

† Rhodopeptin was originally called as Mer-N1033 in the PCT International Patent Applications (W/O 95/26978).

Fig. 1. Structures of rhodopeptins.



	R_1	R_2	R_3
Rhodopeptin C1	$-(CH_2)_3NH_2$	$-CH(CH_3)-CH_3$	$-(CH_2)_6CH(CH_3)-CH_2CH_3$
Rhodopeptin C2	$-(CH_2)_3NH_2$	$-CH(CH_3)-CH_2CH_3$	$-(CH_2)_6CH(CH_3)-CH_2CH_3$
Rhodopeptin C3	$-(CH_2)_3NH_2$	$-CH(CH_3)-CH_3$	$-(CH_2)_8CH(CH_3)-CH_3$
Rhodopeptin C4	$-(CH_2)_3NH_2$	$-CH(CH_3)-CH_3$	$-(CH_2)_8CH(CH_3)-CH_2CH_3$
Rhodopeptin B5	$-(CH_2)_4NH_2$	$-CH(CH_3)-CH_3$	$-(CH_2)_9CH(CH_3)-CH_3$

Mer-N1033. The strain has been deposited at the National Institute of Bioscience and Human-Technology in Japan with the accession number FERM P-12958.

Fermentation

A fragment of a slant culture of strain Mer-N1033 was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of a seed medium. The medium was composed of soluble starch 2%, glucose 2%, soybean meal 2%, yeast extract 0.5%, sodium chloride 0.25%, calcium carbonate 0.32%, and 0.2% metal salt solution containing 0.25% $CuSO_4 \cdot 5H_2O$, 0.25% $MnCl_2 \cdot 4H_2O$, 0.25% $ZnSO_4 \cdot 7H_2O$, and the pH of the medium was adjusted to 7.4 before sterilization. The seed culture was incubated on a rotary shaker at 28°C for 72 hours. For the production of the antibiotics, the 500 ml of seed culture was transferred to a 30-liter jar fermentor containing 15 liters of the same medium. The culture was incubated at 28°C for 72 hours under an aeration rate of 7.5 liters per minute and an agitation of 250 rpm.

The antibiotics present in the methanol extract of the bacterial pellet or in preparations obtained during the purification were detected by the conventional dilution method in Eagle's MEM medium (Nissui) supplemented by glucose 2%, L-glutamine 0.25% and L-asparagine 0.1%, using *Candida albicans* ATCC 10231 as the test organism.

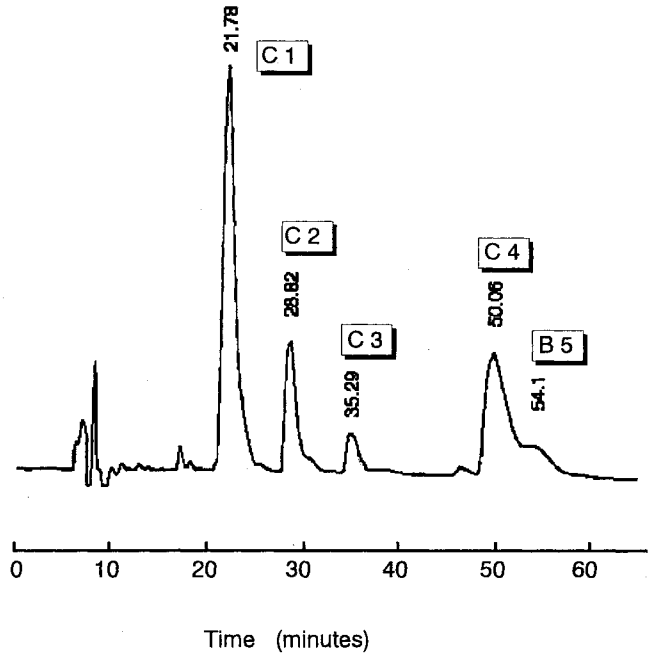
Isolation

The bacterial pellet was obtained by centrifugation of the culture broth (13 liters) and was soaked in 10 liters of methanol at room temperature for 4 hours to extract rhodopeptins. After the removal of the bacterial pellet by centrifugation, the methanol extract was concentrated *in vacuo* to give an aqueous residue. Then, 500 ml of distilled water and 500 ml of ethyl acetate were added to the aqueous residue and mixed vigorously. Rhodopeptins were localized in the interfacial layer between the aqueous layer and the ethyl acetate layer. The interfacial layer was collected and dissolved in 570 ml of 70% methanol. This methanol solution was applied on an MCI-GEL CHP-20P column (Mitsubishi Kasei, 55×170 mm) equilibrated with 70% methanol, and developed with a linear gradient of 70~100% methanol. The biologically active eluates were combined and concentrated *in vacuo* to remove methanol. The concentrate was then lyophilized to give 660 mg of

white powder. The powder was dissolved in 150 ml of methanol and subjected to column chromatography on silica gel (Merck kieselgel 60, 30×380 mm) developed with methanol - 28% NH_4OH (20 : 1). The fractions showing one spot on TLC after staining with ninhydrin were pooled and evaporated to give 95 mg of a mixture of rhodopeptins.

Finally, the rhodopeptins were purified by preparative HPLC (column: YMC-Pack ODS S-343-15, 20×250 mm; mobile phase: acetonitrile - water - trifluoroacetic acid (500 : 500 : 1); flow rate: 7.0 ml/minute; detection: UV at 210 nm). The mixture (18 mg) was dissolved in 4 ml of mobile phase solvent and injected on the HPLC column in four batches. Under these conditions, the retention times of rhodopeptins C1, C2, C3, C4 and B5 were 21.8, 28.8, 35.3, 50.1 and 54.1 minutes, respectively (Fig. 2). The fractions containing the individual rhodopeptins were collected separately and concentrated under reduced pressure to remove acetonitrile and trifluoroacetic acid, and the resulting aqueous suspensions were lyophilized to give pure rhodopeptin C1 (5.0 mg), C2 (2.2 mg), C3 (1.8 mg), C4 (4.0 mg) and B5 (1.7 mg).

Fig. 2. Preparative HPLC pattern of rhodopeptins.



Physico-chemical Properties

The physico-chemical properties of Rhodopeptins are summarized in Table 1. Rhodopeptins are isolated as white powders or colorless solids. The melting points are over 240°C. Rhodopeptins have only UV end absorption. They are soluble in methanol, dimethylsulfoxide, acetic acid, and slightly soluble in H_2O, and are insoluble in ethyl acetate and chloroform. Color reactions are as follows: they give positive reactions to ninhydrin and Rydon-Smith, but negative to iodine and phosphomolybdic acid. On TLC

Table 1. Physico-chemical properties of rhodopeptins.

	C1	C2	C3	C4	B5
Appearance	White powder	Colorless solid	Colorless solid	White powder	Colorless solid
mp	> 240 ℃			> 240 ℃	
Molecular Formula	$C_{25}H_{47}N_5O_4$	$C_{26}H_{49}N_5O_4$	$C_{26}H_{49}N_5O_4$	$C_{27}H_{51}N_5O_4$	$C_{28}H_{53}N_5O_4$
FAB-MS (*m/z*)					
positive	482 $(M+H)^+$	496 $(M+H)^+$	496 $(M+H)^+$	510 $(M+H)^+$	524 $(M+H)^+$
negative	480 $(M-H)^-$	494 $(M-H)^-$	494 $(M-H)^-$	508 $(M-H)^-$	522 $(M-H)^-$
HRFAB-MS (*m/z*)					
Found	482.3708 $(M+H)^+$	496.3853 $(M+H)^+$		510.4016 $(M+H)^+$	
Calcd	482.3706	496.3863		510.4019	
$[\alpha]_D^{26}$ (MeOH)	−15.6° (c 0.14)				
UV in MeOH	end absorption	end absorption	end absorption	end absorption	end absorption
IR (KBr) ν cm^{-1}	3434, 3277, 3083, 2963, 2926, 2855, 1680, 1649, 1553, 1437, 1400, 1381, 1209, 1140, 841, 802, 725	3439, 3277, 3111, 2965, 2928, 2857, 1682, 1651, 1553, 1437, 1400, 1209, 1140, 841, 802, 725	3449, 3297, 3129, 2963, 2932, 2859, 1682, 1655, 1559, 1437, 1399, 1209, 1140, 841, 804, 725	3400, 3279, 3092, 2965, 2926, 2855, 1680, 1649, 1553, 1437, 1400, 1383, 1209, 1142, 841, 802, 725	3432, 3283, 3117, 2963, 2930, 2857, 1680, 1653, 1559, 1437, 1399, 1209, 1140, 839, 804, 725

Table 2. Antimicrobial activities of rhodopeptins.

Microorganism	MIC (μg/ml) [a]					
	C1	C2	C3	C4	B5	amphotericin B
Candida albicans IFM40009	2.5	2.5	2.5	1.25	1.25	0.63
Candida albicans ATCC10231	2.5	5	2.5	2.5	2.5	0.16
Candida kefyr IAM4829	0.63	0.31	0.31	0.31	0.31	0.31
Cryptococcus neoformans TIMM0354	1.25	1.25	0.63	0.63	0.63	1.25
Aspergillus terreus F822	10	10	5	5	5	> 80
Aspergillus fumigatus TIMM0063	> 80	> 80	> 80	> 80	> 80	10
Aspergillus fumigatus IFM41088	> 80	> 80	> 80	> 80	> 80	5
Aspergillus fumigatus IFM4942	> 80	> 80	> 80	> 80	> 80	10
Escherichia coli C600	> 64	> 64	> 64	> 64	> 64	nt [b]
Escherichia coli NIHJ	> 64	> 64	> 64	> 64	> 64	nt
Klebsiella pneumoniae PCI602	> 64	> 64	> 64	> 64	> 64	nt
Enterobacter aerogenes ATCC13048	> 64	> 64	> 64	> 64	> 64	nt
Enterobacter cloacae ATCC13047	> 64	> 64	> 64	> 64	> 64	nt
Salmonella entertidis B-1431	> 64	> 64	> 64	> 64	> 64	nt
Salmonella typhimurium T-287	> 64	> 64	> 64	> 64	> 64	nt
Serratia marcescens IAM1021	> 64	> 64	> 64	> 64	> 64	nt
Proteus mirabilis OM-9	> 64	> 64	> 64	> 64	> 64	nt
Proteus rettgeri GN311	> 64	> 64	> 64	> 64	> 64	nt
Pseudomonas aeruginosa IAM1095	> 64	> 64	> 64	> 64	> 64	nt
Pseudomonas aeruginosa IFO3755	> 64	> 64	> 64	> 64	> 64	nt
Morganella morganii KM-112	> 64	> 64	> 64	> 64	> 64	nt
Bacillus subtilis ATCC6633	> 64	> 64	> 64	> 64	> 64	nt
Micrococcus luteus ATCC9341	> 64	> 64	> 64	> 64	> 64	nt
Staphylococcus aureus FDA209P	> 64	> 64	> 64	> 64	64	nt
Staphylococcus aureus Smith	> 64	> 64	> 64	> 64	16	nt

[a] Broth dilution method using Sabouraud-Dextrose broth for fungi and Cation Supplemented Mueller Hinton broth for bacteria.

[b] nt : Not tested.

(Merck, silica gel 60 F_{254}) developed with *n*-buthanol - acetic acid - H_2O (4 : 1 : 2), all of the rhodopeptin compounds gave a single spot at Rf 0.5.

The details of the structure elucidation will be reported in a separate paper.[1]

Biological Activities

The antifungal activities of rhodopeptins were determined by a two-fold micro-dilution method with Sabouraud-Dextrose broth after incubation at 35°C for 48 hours.[5] As shown in Table 2, rhodopeptins have potent antifungal activity against *Candida* sp., being about as active as amphotericin B, whereas they showed no antibacterial activity.

Cytotoxic activity was measured as follows: mice leukemia L1210 cells were cultured in RPMI-1640 medium (Nissui) supplemented with 10% fetal bovine serum at 37°C in a humidified 5% CO_2 atmosphere. After 48 hours incubation of the cells with the samples, viable cells were

counted using a microculture tetrazolium assay (MTT assay).[6] Rhodopeptin C1 has no growth inhibitory activity at a concentration of 10^{-5} M (4.8 μg/ml).

Discussion

In our screening for antifungal antibiotics we applied the broth dilution method with a *Candida albicans* strain to detect antibiotics produced in a fermentation broth. Using this method, we have isolated a unique family of antifungal antibiotics, the rhodopeptins. We could not detect these antibiotics in the fermentation broth with the ordinary agar diffusion method. We attributed this difference in sensitivity of these methods to poor diffusion of rhodopeptins in the agar medium.

Some cyclic peptides or depsipeptides with antifungal activities have been isolated, for example, echinocandin B[7] and aureobasidins,[8] which are metabolites of fungi. Iturins, produced by a *Bacillus* sp., are cyclicpeptides containing a β-amino acid.[9] Rhodopeptins constitute a group of cyclic tetrapeptides with antifungal activity, which were isolated from *Rhodococcus* sp. Mer-N1033. The following cyclic tetrapeptide antibiotics have been reported: chlamydocin,[10] Cyl-1, Cyl-2,[11] HC-toxin,[12] trapoxin A, trapoxin B[13] and WF-3161.[14] However, the components of these tetrapeptides are all α-amino acids. Therefore, rhodopeptins are a new family of cyclic tetrapeptides.

The mode of action of rhodopeptins, the synthesis of cyclic tetrapeptides analogous to rhodopeptins and the structure-activity relationships of the analogs will be described elsewhere.

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