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YM-266183 and YM-266184, Novel Thiopeptide Antibiotics Produced by *Bacillus cereus* Isolated from a Marine Sponge

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

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Novel antibiotics, YM-266183 (**1**) and YM-266184 (**2**), were found in the culture broth of *Bacillus cereus* QN03323 which was isolated from the marine sponge *Halichondria japonica*. The structures of both antibiotics were determined by several spectroscopic experiments as new thiopeptide compounds. They exhibited potent antibacterial activities against staphylococci and enterococci including multiple drug resistant strains, whereas they were inactive against Gram-negative bacteria.

In the course of the screening for antibacterial substances that have activity against drug-resistant bacteria, novel thiopeptide antibiotics were found from the culture broth of *Bacillus cereus* QN03323 which was isolated from a marine sponge and designated as YM-266183 (**1**) and YM-266184 (**2**) along with thiocillin I (**3**) and II (**4**) (Fig. 1)[1,2]. In this paper, we describe the taxonomy of the producing organism, fermentation, isolation, physico-chemical properties and biological activities of the antibiotics. Structure elucidations of the compounds are reported in the following paper.

Materials and Methods

In situ Observation of Microorganisms by Atomic Force Microscope (AFM)

AFM studies were carried out on a Nanoscope III microscope (Digital Instruments, Santa Barbara, CA). Marine sponges were cut into pieces *ca.* 5 mm in length. They were successively homogenized and suspended in sterile sea water. For dried cell imaging, each suspension was centrifuged, and the pellet was washed and resuspended in sterile sea water. The suspension was mounted on a cover glass coated with poly-L-lysine. After a quick rinse in distilled water and air drying, cells were imaged with AFM by the tapping mode using Si cantilevers.

Isolation of Producing Organism

Marine sponge *Halichondria japonica*, collected at Hoshisuna Beach, Iriomote Island, Okinawa Prefecture, Japan, was used for the isolation of microorganisms. Using the sponge suspension described above, bacteria were isolated on Marine Agar (MA, Difco) and 1/10 MA by the dilution plate method. The plates were incubated at 24°C for one week.

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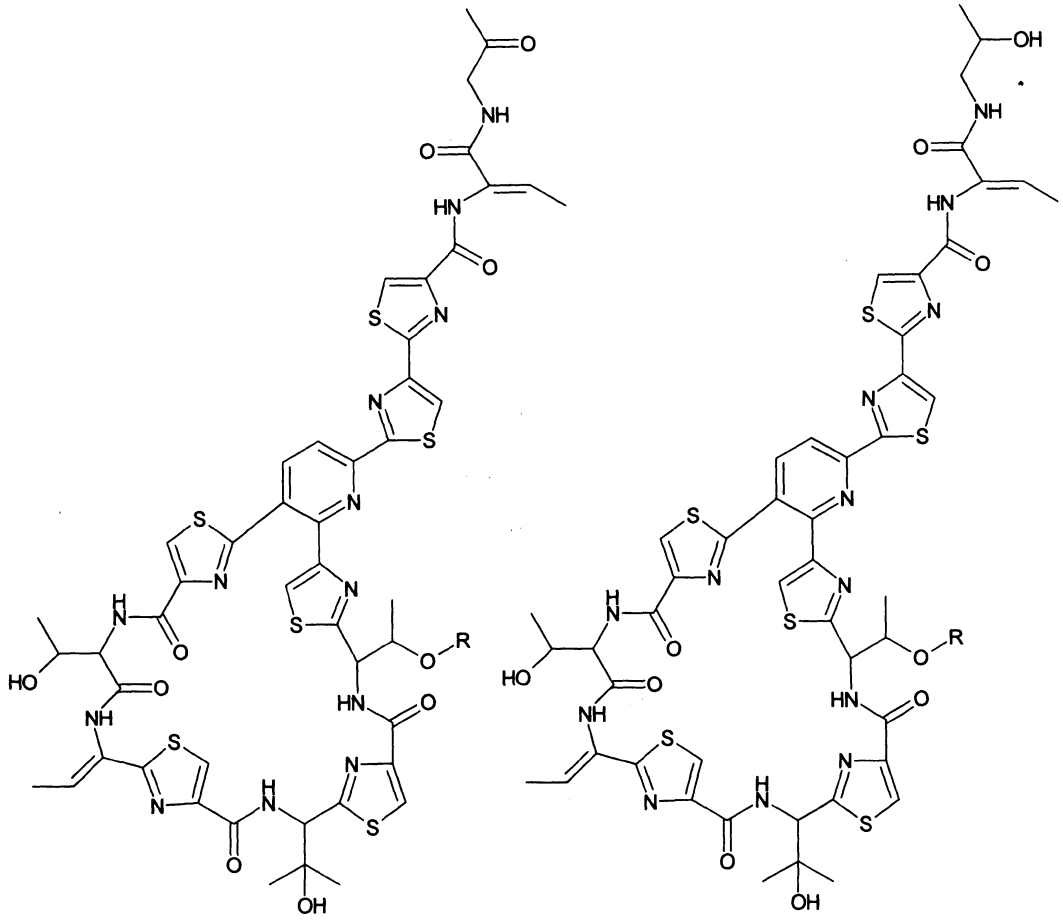
Fig. 1. Structures of YM-266183 (**1**), YM-266184 (**2**), thiocillin I (**3**) and thiocillin II (**4**).



1 R = H
2 R = CH_3

3 R = H
4 R = CH_3

Taxonomic Studies

Morphological properties were observed under an optical microscope (Nikon OPTIPHOT-2). Observation of growth on various media and tests for physiological characteristics were made on the basis of methods of COWAN[3], and CLAUS and BERKELEY[4] during incubation at 10 to 50°C for 21 days unless otherwise noted. Isoprenoid quinones were extracted with chloroform - methanol (2 : 1, vol/vol)[5] and analyzed by mass spectra (LC/ESI-MS and EI-MS). The guanine plus cytosine (G+C) content of the DNA was determined by the method of MESBAH *et al.*[6].

Fermentation

A thawed suspension of strain QN03323 was used to inoculate into a 500-ml Erlenmeyer flask containing 100 ml of a seed medium consisting of glucose 1.0%, potato starch 2.0%, yeast extract 0.5%, Polypepton (Nihon Pharmaceutical Co., Ltd.) 0.5% and $CaCO_3$ 0.4% (pH 7.0). The seed culture was incubated at 28°C for 72 hours on a rotary shaker at 220 rpm. The seed culture (8 ml) was transferred to a 2-liter flask containing 400 ml of the same medium. After 3 days incubation at 28°C, the second seed culture was used to inoculate into a 30-liter jar fermenter containing 18 liters of a production medium consisting of oatmeal 2.0%, meat extract 1.0% (pH 7.5). The fermentation was run for 48 hours at 28°C with an agitation rate of 250 rpm and aeration of 1 vvm. The antibiotic production was monitored by HPLC analysis.

Physico-chemical Properties

IR spectra were recorded on a Perkin Elmer microscope FT-IR spectrometer. Optical rotation was determined on Horiba SEPA-200 polarmeter. HRMALDITOF-MS was measured with Applied Biosystems Voyager ELITE XL time-of-flight mass spectrometer using CHCA (α-cyano-4-hydroxycinnamic acid) as matrix.

Antimicrobial Activity

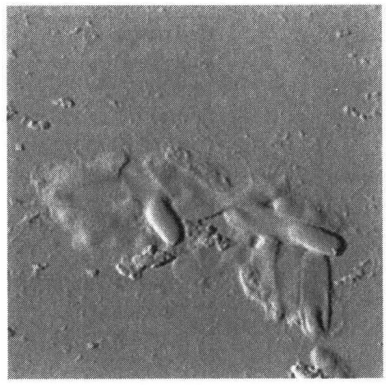
Antimicrobial spectra were determined by the serial agar dilution method on Mueller-Hinton agar (Difco). Approximately 10^4 CFU per spots were inoculated onto agar plates that contained two-fold serial dilutions of antibiotics. Minimal inhibitory concentrations (MICs) were indicated as the lowest concentration of antibiotics that completely inhibited visible growth after incubation for 24 hours. Methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant *Streptococcus epidermidis* (MRSE) were incubated at 32°C, and the others were incubated at 37°C.

Results and Discussion

Observation of Microorganisms in Sponge Samples

An atomic force microscope (AFM) was used for the detection of microorganisms and evaluation of marine sponges as microbial hosts. The numbers and morphological properties (*e.g.*, cell surface structures and flagella) of living bacteria were observed without pretreatments of samples such as fixation or staining. Based on the method described above, *Halichondria japonica* was selected as one of promising sources for isolation of symbiont microorganisms. Fig. 2 shows AFM images of bacterial cells in the sponge *Halichondria japonica* which was used for the isolation of microorganisms.

Fig. 2. AFM images of microorganisms inhabiting the sponge.



Taxonomy of the Producing Strain

The cultural and physiological characteristics are listed in Table 1. Colony of strain QN03323 was opaque, circular with irregular circumference. No diffusible pigment was produced. By optical and electron microscopic observation, strain QN03323 was Gram-positive, slender rods, motile, usually 0.9 to 1.7 μm in width, 4.0 to 7.0 μm in length (Fig. 3). Spores formed under aerobic conditions were elliptical, mostly in central to terminal position. Several amino acids were required for the growth. Vitamins were not required. Nitrite formation from nitrate, Voges-Proskauser reaction, hydrolysis of starch, urease, oxidase and catalase activities were positive. Denitrification, methyl red test (glucose peptone broth), indole production, H_2S production were negative. Citrate, potassium nitrate and ammonium sulfate were not utilized as a sole nitrogen source. It was facultatively anaerobic. The temperature range for growth was 10 to 45°C with an optimum temperature range of 28 to 40°C. The pH range for growth was 5.0 to 9.0 with an optimum pH range for growth of 6.0 to 8.0. Acid formation was observed from D-fructose, D-glucose, D-mannose, maltose, sucrose, trehalose, glycerol and starch. The major isoprenoid quinone was determined to be unsaturated menaquinone with seven isoprenoid units. The GC-content of the DNA was determined to be 36.4 mol%.

Based on cultural and microscopic characteristics described above, the strain QN03323 was identified as a bacterium of the genus *Bacillus*. Then, the characteristics of the strain QN03323 were compared with the known species of *Bacillus* described in BERGEY's Manual of Determinative Bacteriology (9th Ed.)[7], BERGEY's Manual of Systematic Bacteriology (Vol. 2) and other reports. As a results the strain QN03323 was presumed to be closest to *Bacillus cereus* Frankland and Frankland. Therefore, this strain was designated as *Bacillus cereus* QN03323. The strain has been deposited in the National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan with the accession No. FERM BP-7864.

Fermentation

Fig. 4 shows the typical time course of YM-266183 and

Table 1. Morphological and physiological characteristics of strain QN03323.

Cell shape	Non-vacuolated straight rod
Cell size	0.9 – 1.7 x 4.0 – 7.0 μm
Motility	Positive
Spore	Ellipsoidal
Position of spore	Central to terminal
Gram stain	Positive
Reduction of nitrate	Positive
Denitrification	Negative
Methyl red test	Negative
Voges-Proskauser test	Positive
Production of:	
Indole	Negative
H_2S	Negative
Utilization of:	
Citrate	Negative
KNO_3	Negative
$(NH_4)_2SO_4$	Negative
Growth factor requirement	Positive
Urease	Positive
Oxidase	Positive
Catalase	Positive
OF-test	Not reactive
Range of growth:	
pH	5.0 – 9.0
(Optimum)	(6.0 – 8.0)
Temperature	10 – 45°C
(Optimum)	(28 – 40°C)
Acid formation from	D-fructose, D-glucose, D-mannose, Maltose, Sucrose, Trehalose, Glycerol, Starch
Hydrolysis of starch	Positive
Liquefaction of gelatin	Positive
Tolerance to NaCl	0 - 6%
Mol% G + C of DNA	36.4

Fig. 3. Optical micrograph of strain QN03323.

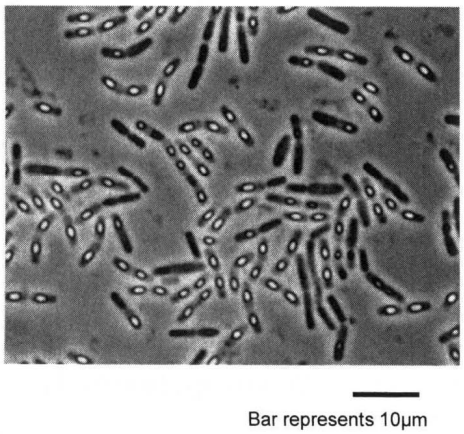
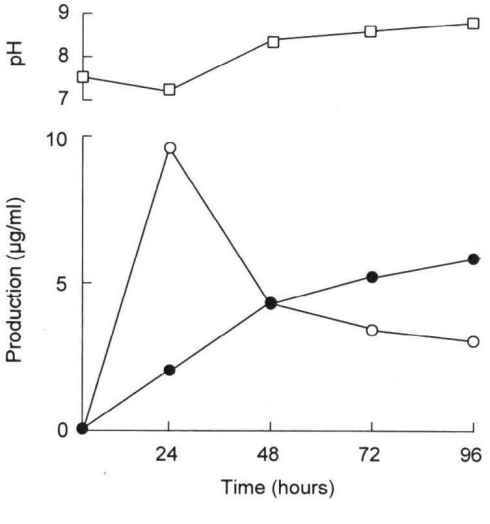


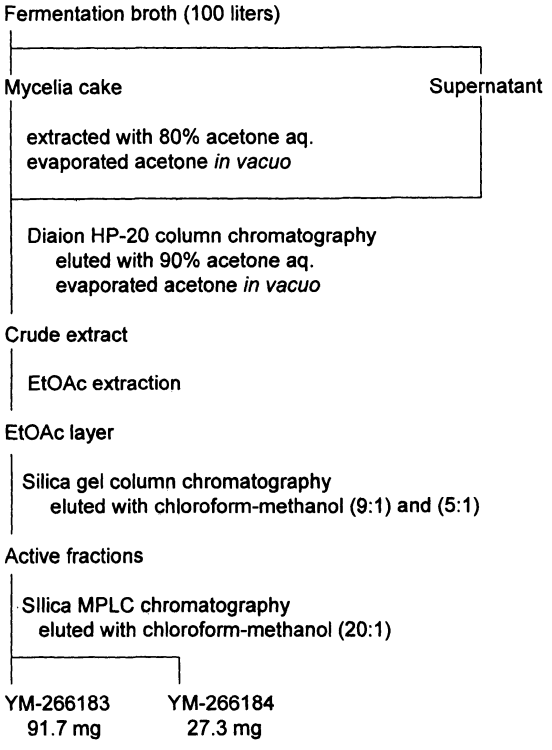
Fig. 4. Time course of YM-266183 (**1**) and YM-266184 (**2**) production.



□ pH, ● **1**, ○ **2**

YM-266184 production by *Bacillus cereus* QN03323 in a 30-liter jar fermenter. The production of YM-266183 started at 24 hours after inoculation, then increased and reached a maximum (5.6 μg/ml) at 96 hours. In contrast, the production of YM-266184 showed a maximum (9.6 μg/ml) at 24 hours, then decreased.

Fig. 5. Isolation and purification procedure of YM-266183 and YM-266184.



Isolation and Physico-chemical Properties

The purification procedures of YM-266183 and YM-266184 are outlined in Fig. 5. About 100 liters of the culture broth was centrifuged. The mycelia cake was extracted with 80% acetone solution (10 liters), and evaporated under reduced pressure to remove acetone. The residue and the supernatant were applied on Diaion HP-20 column, washed with water and 30% acetone solution, and eluted with 90% acetone solution (30 liters). The elution was concentrated *in vacuo* to remove acetone, and extracted with ethyl acetate (10 liters) twice. The extraction was dried up, subjected to silica gel column (i.d. 65×130 mm), and eluted with chloroform - methanol (9 : 1) and (5 : 1). The active fraction was applied on silica gel medium pressure liquid chromatography using chloroform - methanol (20 : 1) to collect YM-266183 (91.7 mg) and YM-266184 (27.3 mg).

Physico-chemical properties of YM-266183 and YM-266184 are summarized in Table 2. The details of the structure elucidation studies of YM-266183 and YM-266184 will be described in the succeeding paper[8].

Antibacterial Activity

Antibacterial spectra of **1** and **2** are shown in Table 3. MICs were determined by the serial agar dilution method using Mueller-Hinton medium. Both compounds were active against Gram-positive bacteria including MRSA, MRSE and vancomycin-resistant Enterococci (VRE), whereas they were inactive against Gram-negative bacteria, fungi and yeasts. The MICs of **1** and **2** were similar to those

Table 2. Physico-chemical properties of YM-266183 (**1**) and YM-266184 (**2**).

	1	**2**
Appearance	Colorless powder	Colorless powder
Molecular formula	$C_{48}H_{47}N_{13}O_{10}S_6$	$C_{49}H_{49}N_{13}O_{10}S_6$
HRMALDITOF-MS (*m/z*)		
Found:	1180.1795 $(M+Na)^+$	1194.1933 $(M+Na)^+$
Calcd:	1180.1791	1194.1948
UV λ_{max} nm (ε) (in MeOH)	213 (79833), 290 (sh), 344 (14463)	215 (100963), 290 (sh), 346 (29349)
IR ν_{max}^{KBr} cm^{-1}	3385 (OH), 1662 (CO), 1534, 1481, 754	3399 (OH), 1661(CO), 1533, 1481, 753
$[\alpha]_D^{25}$	64.7° (*c* 0.37, MeOH)	60.9° (*c* 0.15, MeOH)

Table 3. Antimicrobial activities of YM-266183 (**1**) and YM-266184 (**2**).

Test organisms	MIC (μg/ml)					
	1	**2**	thiocillin I	thiocillin II	thiostrepton	vancomycin
Staphylococcus aureus FDA 209P	0.025	0.025	0.05	0.013	0.025	0.39
S. aureus CAY 27701 (MRSA)	0.78	0.39	0.78	0.2	0.1	0.78
Streptococcus epidermidis CAY 4402 (MRSE)	1.56	0.2	0.2	0.025	0.1	1.56
Enterococcus faecalis CAY 04_1	0.1	0.025	0.05	0.013	0.025	0.78
E. faecium CAY 09_1	0.2	0.05	0.05	0.025	0.025	0.78
E. faecium CAY 09_2 (VRE)	0.025	0.025	0.025	0.006	0.013	>100
Bacillus subtilis ATCC 6633	1.56	1.56	1.56	0.39	0.025	0.2
Escherichia coli JCM 5491	>100	>100	>100	>100	>100	>100

of thiocillin I and II.

Acknowledgement

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