Kalimantacins A, B and C, Novel Antibiotics from *Alcaligenes* sp. YL-02632S

I. Taxonomy, Fermentation, Isolation and Biological Properties

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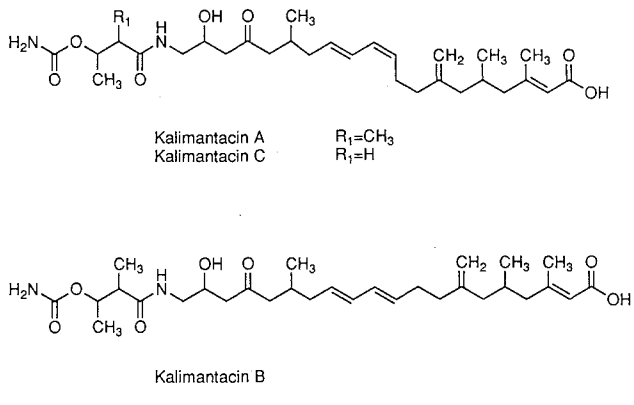
Novel antibacterial antibiotics, kalimantacins A, B and C, have been isolated from the fermentation broth of *Alcaligenes* sp. YL-02632S. In this paper, the taxonomy of the producing strain, fermentation, isolation and biological activities of kalimantacins are reported. Kalimantacins inhibit the growth of *Staphylococcus aureus* and *S. epidermidis* including multiple-drug resistant strains.

In the course of a screening program for a novel antibiotic, kalimantacins were found from the culture broth of *Alcaligenes* sp. YL-02632S (Fig. 1). The antibiotics exhibited the antibacterial activities against *S. aureus* and *S. epidermidis* including multiple-drug resistant strains. The present paper deals with the taxonomy, fermentation, isolation and biological activities of the new antibiotics.

Fig. 1. Structures of kalimantacins A, B and C.



Materials and Methods

Isolation and Classification of Producing Organisms

The strain, YL-02632S, was freshly isolated from a soil sample collected from West Kalimantan, Indonesia. Soil samples were dried at room temperature overnight and inoculated in the isolation medium (GB-medium). GB-medium consisted of L-asparagine 0.1%, $CaCl_2$ 0.1%, agar 1.5%, in humus soil extract solution at pH 8.0 before sterilization. Twenty-five μg/ml of cefotetan (Yamatetan: Yamanouchi Pharmaceutical Co., Ltd.), and 25 μg/ml of Penicillin G potassium (Wako Pure Chemical Industries, Ltd.) were supplied to the medium as a selective pressure for Gram-negative bacteria. Nystatin (50 μg/ml) and cycloheximide (50 μg/ml) were also added to the medium to suppress the growth of fungi. The plates were incubated at 27°C for 1 month and checked at regular intervals of 3~5 days. Colonies grown on the medium were classified preliminarily and picked to YS-agar slants (yeast extract 0.2%, potato starch 1.0%, agar 1.5%, pH 7.6 before sterilization).

Observation of growth on various media and tests for physiological characteristics were made on the basis of methods of COWAN[1], CHRISTENSEN and COOK[2], DWROKI and GIBSON[3] and GILARDI[4] during incubation at 10~15°C for 21 days unless otherwise mentioned. Deoxyribonucleic acid extracts were prepared by the method discribed in "Genetic Manipulation of Streptomyces"[5]. The guanine-plus-cytosine content of the

deoxyribonucleic acids of strain YL-02632S was determined by the method of MARMUR[6], and MURMUR and DOTY[7].

Fermentation

Stock cultures were maintained as frozen whole broths at −80°C in a final concentration of 10% dimethyl sulfoxide. A 500-ml Erlenmeyer flask containing 100 ml of seed medium was inoculated with 2 ml of stock culture. The flask was incubated at 28°C on a rotary shaker (220 rpm; 5 cm stroke) for 48 hours. The seed medium consisted of glucose 1.0%, yeast extract 0.1%, meat extract 0.1%, NZ-amine type A 0.2% in distilled water. The pH was adjusted to 7.3 before sterilization. The seed culture broth (1.2 liters) was used to inoculate a 30-liter fermentor containing 18 liters of a production medium consisting of glucose 2.0%, yeast extract 0.2%, meat extract 0.2%, NZ-amine type A 0.4%, 5 mM L-aspartic acid in distilled water. The pH was adjusted to 7.0 before sterilization. The fermentation was carried out for 40 hours at 28°C with an air flow of 18 liters per minute and an agitation rate of 300 rpm. The antibiotic production was monitored by HPLC analysis.

Isolation

The purification procedures of kalimantacins are outlined in Fig. 3. The organic solvents and reagents used in the purification were purchased from Kanto Chemical Co., Inc. The columns of preparative and analytical HPLC were purchased from Simadzu Techno-Research, Inc.

Determination of the Antimicrobial Activities

Minimum inhibitory concentrations (MICs) were determined by a conventional agar dilution method using Mueller-Hinton medium for Gram-positive and Gram-negative organisms. MICs were expressed in terms of μg/ml after overnight incubation at 37°C.

Cytotoxicity of Kalimantacin A

The cytotoxicity of the compound against two cell lines (P388 mouse leukemic cells and HeLa S3 cells) was determined using the trypan blue dye exclusion method. P388 cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum and 20 mM HEPES buffer. HeLa S3 cells were cultured in HAM's F12 supplemented with 10% fetal bovine serum and 20 mM HEPES buffer. The cells were incubated in the presence or absence of the compound at 37°C for 72 hours in a humidified atmosphere containing 5% CO_2. IC_{50} was determined by plotting the logarithm of the drug concentration versus the growth rate of the treated cells.

Results and Discussion

Taxonomic Studies of the Producing Strain

The strain YL-02632S was isolated from a soil sample collected from West Kalimantan, Indonesia. Colonies of YL-02632S were semi-transparent, grayish white, circular, and convex on nutrient agar. By light and electron microscopic observation, the strain YL-02632S was Gram-negative rods, 0.6~1.0 μm in width, 1.8~2.2 μm in length, motile with peritrichous flagella. The strain YL-02632S did not require any growth factors and did not form fruiting bodies, intracellular granules, photosynthetic pigments, or spores of microcyst. The other cultural and physiological characteristics are listed in Table 1. The key characters of the strain YL-02632S were as follows: Gram-negative; aerobic rods; motile with peritrichous flagella; non-pigmented; oxidase positive; catalase-positive; indole not produced; chemoorganotrophic; alkali production; above 60 mol% of DNA content. Based on taxonomic properties described above, the strain YL-02632S was placed in the genus *Alcaligenes*[8].

A large number of taxonomical studies on *Alcaligenes* species have been published. However, many of the type strains were poorly characterized and sometimes not deposited in culture collections. Therefore, the taxonomical properties of YL-02632S were compared with those of the authentic species such as *A. faecalis*, *A. denitroficans* subsp. *denitroficans*, *A. denitroficans* subsp.

Table 1. Morphological and physiological characteristics of the strain YL-02632S.

Cell size (μm)	0.6~1.0×1.8~2.2
Motility with peritrichous flagella	Positive
Oxidase reaction	Positive
Catalase reaction	Positive
OF-test	Not reactive
Peptonization of milk	Positive
Coagulation of milk	Positive
Haemolisis	Negative
Gelatin hydrolysis	Positive
Esculin hydrolysis	Negative
Tween 80 hydrolysis	Negative
Starch hydrolysis	Negative
Indole production	Negative
H_2S production	Negative
Anaerobic growth	Negative
Accumiration of Poly-β-hydroxybutyrate	Negative
Tolerance to NaCl	0~2%
Alkali production in litmus milk	Positive
Arginine dihydrolase	Negative
Lysine decarboxylase	Negative
Ornithine decarboxylase	Negative
Urease reaction	Negative
Acylamidase reaction	Negative
Voges-Proskauer reaction	Negative
Methyl red reaction	Negative
Nitrate reduction	Positive
Dentrification	Negative
Citrate utilization	Negative
Mol% G+C of DNA	62.8

xylosoxydans, and "*A. odrans* var. *viridans*". As the results, the strain YL-02632S were found to be similar to *A. denitroficans* subsp. *denitroficans*. But there are some differences in physiological properties and mol% G+C of DNA. Tests for nitrate reducing activity and hydrolytic activity of gelatin are positive for the strain YL-02632S; those are negative for *A. denitroficans* subsp. *denitroficans*. Mol% G+C of DNA of YL-02632S is 62.8; 63.9 for *A. denitroficans* subsp. *denitroficans*. From the results described above, the strain YL-02632S was designated *Alcaligenes* sp. YL-02632S. The type strain, YL-02632S, was deposited at National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, with the accession number. FERM-12694.

Fermentation

Alcaligenes sp. YL-02632S was grown in a 30-liter fermentor for 48 hours at 28°C. A typical time course for the production of Kalimantacin-A is presented in Fig. 2. Antibiotic production followed the cell growth and reached a maximum at 40~48 hours.

Isolation and Purification Subsp.

The purification procedures of kalimantacins are outlined in Fig. 3. The fermentation broth of *Alcaligenes* sp. YL-02632S (18 liters) was centrifuged at 8,000 rpm for 10 minutes. The supernatant was subjected to DIAION HP-20 column chromatography (500 ml). After washing with water (5 liters) and then 25% aqueous acetone (5 liters), the active components were eluted with 55% aqueous acetone (5 liters). The elution was concentrated *in vacuo* to remove acetone, and extracted with ethyl acetate (3 liters) at pH 5.0. The organic layer was extracted with 0.3% $NaHCO_3$ solution adjusted to pH 9.0 four times and again transferred to an ethyl acetate layer at pH 5.0. The extract was evaporated *in vacuo* to yield a brown syrup (278 mg). This crude product was subjected to silica gel column chromatography (10 × 370 mm) using $CHCl_3$ - MeOH (96 : 4) as solvent. The fractions exhibiting antibacterial activities against

Fig. 2. Time course of kalimantacin A fermentation.

△ pH, ○ dry cell weight, ▲ glucose, ● kalimantacin A.

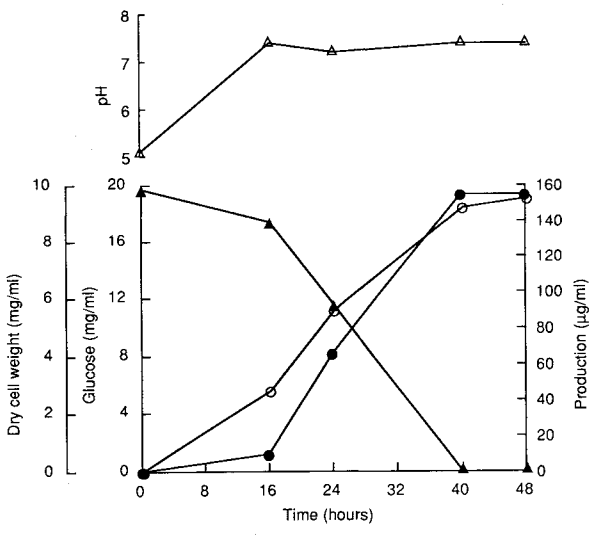


Fig. 3. Isolation and purification procedure of kalimantacins.

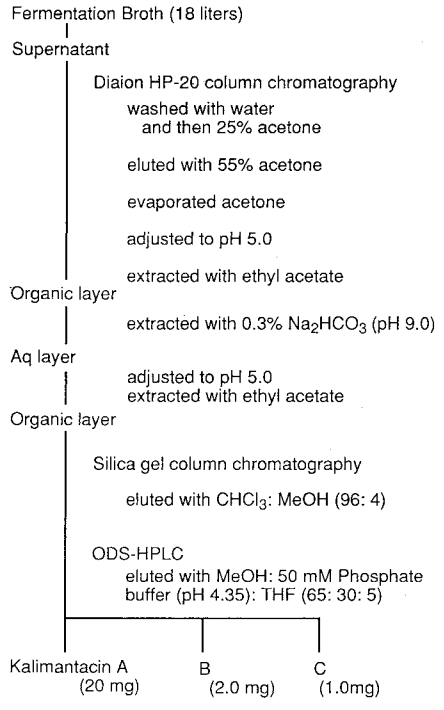
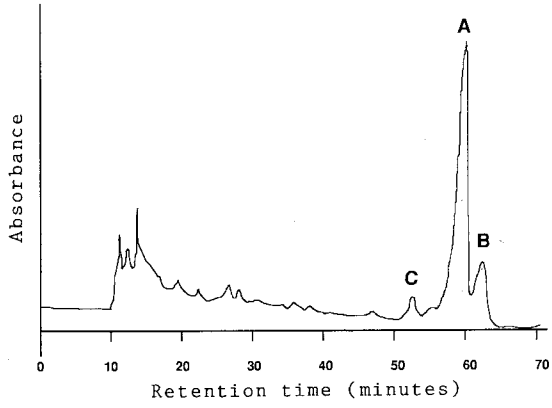


Fig. 4. HPLC chart of kalimantacins.

A: kalimantacin A, B: kalimantacin B, C: kalimantacin C.



HPLC conditions

Column:	Shimadzu STR-ODS (20 × 250 mm)
Mobile phase:	methanol - 50 mM phosphate buffer (pH 4.35) - tetrahydrofuran (65 : 30 : 5)
Flow rate:	8 ml/minute
Detection:	UV 230 nm

Table 2. Antimicrobial spectra of kalimantacins.

Test organisms	MIC (μg/ml)						
	Kalimantacin A	Kalimantacin B	Kalimantacin C	Vancomycin	Flomoxef	Cefmetazole	IPM/CS*
Staphylococcus aureus FDA 209P	0.2	0.78	0.2	0.39	0.39	0.78	0.013
S. aureus No. 5 (MRSA)**	0.2	1.56	0.2	0.78	>100	100	100
S. epidermidis HD866	0.2	0.78	0.39	1.56	0.78	1.56	<0.006
S. epidermidis No. 17 (MRSE)**	0.2	0.78	0.39	0.78	25	25	25
Bacillus subtilis ATCC 6633	>100	>100	>100	0.78	0.39	0.78	0.013
Salmonella enteritidis 1891	3.13	12.5	3.13	>100	0.1	0.39	0.2
Escherichia coli 0~1	25.0	50	25.0	>100	0.025	0.39	0.1
Pseudomonas aeruginosa ATCC 8689	>100	>100	>100	>100	>100	>100	0.98
Klebsiella pneumoniae ATCC 10031	3.13	12.5	3.13	>100	0.05	0.39	0.1

* Imipenem cilastatin sodium.
** Clinical isolated strains, resistant to β-lactams, macrolides and quinolones.

S. aureus FDA 209P were collected and evaporated *in vacuo*. The crude syrup was dissolved in MeOH and applied to preparative HPLC (column; Simadzu STR-ODS, 20 × 250 mm, flow rate; 8 ml/minute). The column was developed with methanol - 50 mM phosphate buffer (pH 4.35) - tetrahydrofuran (65 : 30 : 5) to collect each peak fraction separatively. The HPLC profile of kalimantacins is shown in Fig. 4. The yields of pure kalimantacins A, B, and C were 20, 2.0 and 1.0 mg, respectively.

Biological Activities of Kalimantacins

Antimicrobial spectra of kalimantacins A, B, and C are shown in Table 2. MICs were determined by the serial agar dilution method using Mueller-Hinton medium. Kalimantacin A showed the antimicrobial activity against *S. aureus* and *S. epidermidis* including multiple-drug resistant strains, but are inactive against most Gram-negative bacteria and fungi. The MICs of kalimantacin B were less than that of kalimantacin A, and kalimantacin C was almost comparable in activity to kalimantacin A.

Cytotoxic activity of kalimantacin A was examined against P388 and HeLa S3 cells *in vitro*. When the cells were exposed to the antibiotic for 3 days, the IC_{50} values were 35 and 50 μg/ml respectively (the IC_{50} values of Mitomycin C were 0.010 and 0.017 μg/ml respectively).

Acknowledgments

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