Isolation of Cytotoxic Substance, Kalafungin from an Alkalophilic Actinomycete, *Nocardiopsis dassonvillei* subsp. *prasina*

Hiroshi TSUJIBO,[a] Takashi SAKAMOTO,[a] Katsushiro MIYAMOTO,[a] Genjiro KUSANO,[a] Masaru OGURA,[b] Tōru HASEGAWA[c] and Yoshihiko INAMORI*,[a]

Osaka University of Pharmaceutical Sciences,[a] Kawai, Matsubara-shi, Osaka 580, Japan, Yamasa Shoyu Co., Ltd.,[b] 10–1, Araoi-cho, 2-chome, Chosi-shi, Chiba 288, Japan and Institute for Fermentation Osaka,[c] Jusohonmachi, 2–17–85, Yodogawa-ku, Osaka 532, Japan. *Received January 10, 1990*

An alkalophilic actinomycete, strain OPC-553 regarded as *Nocardiopsis dassonvillei* subsp. *prasina*, produced the cytotoxic substance, TS-1, which showed a marked inhibitory activity against L5178Y mouse leukemic cell *in vitro*. The cytotoxicity of TS-1 on this cell was very strong and its ID_{50} was 0.018 μg/ml. Through direct comparison of its spectral data with those of an authentic sample, TS-1 was identified as the antifungal antibiotic, kalafungin, already isolated from the culture broth of *Streptomyces tanashiensis*. However, the isolation of kalafungin from an alkalophilic actinomycete and its cytotoxicity are reported for the first time in this paper.

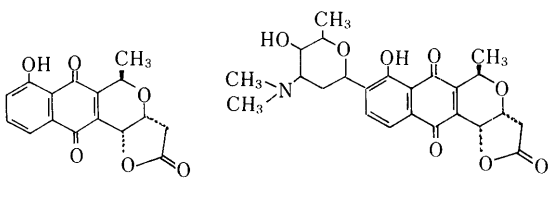
Keywords alkalophilic actinomycete; *Nocardiopsis dassonvillei* subsp. *prasina*; kalafungin; antifungal antibiotic; cytotoxicity; L5178Y mouse leukemic cell; *Streptomyces tanashiensis*; Horikoshi medium

So far, screening tests for the biologically active substances produced by alkalophilic actinomycetes have been carried out in our laboratory. The authors previously reported that an alkalophilic actinomycete regarded as *Nocardiopsis dassonvillei* OPC-15 produced 1,6-dihydroxyphenazine and N-oxidation of this antibiotic was preceeded by a temperature shift from 27 °C to 4 °C.[1] Subsequently, a large number of alkalophilic actinomycetes have been isolated from soil samples according to the method of Horikoshi,[2] in order to obtain novel biologically active substances. Among them, an alkalophilic actinomycete strain, OPC-553, regarded as *Nocardiopsis dassonvillei* subsp. *prasina* was found to produce the cytotoxic substance, TS-1. Through direct comparison of its spectral data with those of an authentic sample, TS-1 was identified as kalafungin, the antifungal antibiotic already isolated from the culture broth of *Streptomyces tanashiensis* by Bergy *et al.*[3] (Chart 1). However, there is no report on the production of kalafungin by alkalophilic actinomycetes or on its cytotoxicity.

In this paper, isolation, identification and cytotoxicity on L5178Y mouse leukemic cell of TS-1 (kalafungin) produced by an alkalophilic actinomycete, strain OPC-553, regarded as *N. dassonvillei* subsp. *prasina*, were described.

Materials and Methods

Isolation Method for Alkalophilic Actinomycetes The isolation of alkalophilic actinomycetes was carried out according to the method of Horikoshi.[2] A small amount of soil was suspended in 100 ml of sterilized H_2O. One hundred μl aliquots of the suspension were spread on agar plates containing 1.0% glucose, 0.5% polypeptone (Daigo Eiyo), 0.5% yeast extract (Difco), 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 1.0% Na_2CO_3 and 2% agar (Difco) in distilled H_2O, pH 10.0. Sodium carbonate was sterilized separately and then added to the medium. The plates were incubated at 27 °C for 7—10 d. An alkalophilic actinomycete, strain OPC-553, showing cytotoxic activity against L5178Y mouse leukemic cell *in vitro* was isolated from among 200 colonies.



kalafungin lactoquinomycin-A

Chart 1

Taxonomic Studies on Strain OPC-553 For the cultural and physiological characterization of strain OPC-553, the methods and media recommended by the International Streptomyces Project (ISP)[4] and those recommended by Waksman[5] were used. The pH of each medium was adjusted to 10.0 by adding a sterile 1 M Na_2CO_3 solution after autoclaving. The growth-permissive pH and temperature were determined on agar plate (1.0% glucose, 0.5% polypepton, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$ and 1.5% agar) with the pH adjusted to 6—10.5 with 1 M Na_2CO_3. The morphology of the aerial mycelia of strain OPC-553 grown on yeast extract–malt extract agar (ISP medium 2) with the pH adjusted with 1 M Na_2CO_3 were studied by a light microscope. For scanning microscopy, an agar block containing numerous spores on ISP medium 2 (pH 10.0) was gradually dehydrated with an increasing amount of EtOH and finally dried by the critical point method. Each specimen was coated with evaporated gold and examined with a JEOL T-20 scanning electron microscope. Diaminopimelic acid isomer in cells was analyzed by the method of Hasegawa *et al.*[6] Whole-cell sugar was analyzed using the high performance liquid chromatography (HPLC) system.[7] Mycolic acid analysis was performed by the method of Yano *et al.*[8] Mycolic acid of *Nocardia asteroides* 2103 was used as a standard. Phospholipid analysis was determined by the method of Minnikin *et al.*[9] Isoprenoid quinone analysis was performed by Collins *et al.*[10]

Production of Cytotoxic Substance, TS-1 A loopful of spores of strain OPC-553 was transferred to a 500 ml flask containing a medium of: 1% glucose, 0.5% polypeptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 1.0% Na_2CO_3 (pH 10.0) and this flask was incubated with reciprocal shaking (120 rpm, amplitude, 7 cm) for 7 d.

Assay of Growh-Inhibitory Activity against L5178Y Cell The inhibitory action on the growth of L5178Y mouse leukemic cells was determined as described previously.[11] Briefly, the leukemic cells were grown in RPMI 1640 medium (Nissui Co., Ltd.) supplemented with 10% bovine serum at 37 °C. An appropriate amount of sample in serial half-$\log_{10}$ dilutions was added to 9 volumes of a suspension of cells in a logarithmic phase (1.6×10^5 cells per ml). After 2 d of incubation at 37 °C, the number of cells per tube were counted with a microcell counter, Sysmex CC-110, and a 50% inhibitory dose (ID_{50}) was obtained graphically.

Isolation and Purification of Cytotoxic Substance, TS-1 The culture broth (14 l), after being adjusted to pH 7.0 with 6 N HCl, was extracted three times with AcOEt. An AcOEt layer, after being dried with Na_2SO_4 overnight, was evaporated to dryness under reduced pressure. AcOEt extract was chromatographed on silica gel with $CHCl_3$–MeOH (8 : 2) as an eluent to give the active fraction (Fr-I-A). The active fraction was subjected to gel filtration on a Sephadex LH-20 column using MeOH as an eluent to obtain the active fraction (Fr-I-B). Fr-I-B was chromatographed on preparative thin layer chromatography (TLC) (KIESELGEL 60 F-254, DC-Fertigplatten) with $CHCl_3$–MeOH (8 : 2, v/v) as an eluent to give the active fraction (Fr-I-C). The active fraction (Fr-I-C) was further purified by preparative HPLC. Apparatus, high-performance liquid chromatograph (Toyo Soda HLC-803 Series A); column, Cosmosil

5 SL (10 × 250 mm); detector, UV 265 nm; mobile phase, $CHCl_3$–MeOH (98 : 2, v/v), $CHCl_3$–*n*-hexane–MeOH (63 : 35 : 2, v/v); flow rate, 3 ml/min; temperature, ambient. One peak was obtained (t_R: 5 min 48 s, $CHCl_3$–MeOH = 98 : 2, v/v, 7 min 24 s, $CHCl_3$–*n*-hexane–MeOH = 63 : 35 : 2, v/v). The active principle with cytotoxicity was designed as TS-1 (5.42 mg). TS-1 (Kalafungin): mp 163—165 °C (dec.). UV_{max}^{MeOH} nm (log ε): 258 (4.53). MS *m/z*: 300.0653 (M^+, Calcd for $C_{16}H_{12}O_6$; 300.0672). ^{1}H-NMR (δ ppm, $CDCl_3$): 1.52 (d, J = 7.0 Hz, 1-CH_3), 2.57 (d, J = 18.0, 11-H_1), 2.97 (dd, J = 18.0, 4.5 Hz, 11-H_2), 4.69 (dd, J = 5.1, 2.9 Hz, 3-H), 5.10 (q, J = 7.0 Hz, 1-H), 5.27 (d, J = 2.9 Hz, 4-H), 7.71 (d, J = 7.8 Hz, 6-H), 7.78 (dd, J = 7.8, 3.5 Hz, 7-H), 7.91 (dd, J = 7.8, 3.5 Hz, 8-H), 11.8 (br s, 9-OH).

Results

Taxonomic Studies on Strain OPC-553 Taxonomic studies on strain OPC-553 have been done according to the usual methods.[4-10] On the basis of the results of microscopic and chemotaxonomic studies, OPC-553 was regarded as *Nocardiopsis dassonvillei* subsp. *prasina*.[12]

Isolation, Purification and Identification of Cytotoxic Substance, Kalafungin (TS-1) The cytotoxic principle was purified by extraction with AcOEt followed by a combination of column chromatography, preparative TLC and HPLC. The cytotoxic substance isolated was named as TS-1. All spectral data of TS-1 were very similar to those of kalafungin, an antifungal antibiotic already isolated from the culture broth of *Streptomyces tanashiensis* by Bergy *et al.*[3] Through direct comparison of its spectral data with those of an authentic sample, TS-1 was identified as kalafungin. Next, the inhibitory effect of kalafungin on L5178Y mouse leukemic cell *in vitro* was investigated. As shown in Fig. 1, kalafungin markedly inhibited the growth of L5178Y mouse leukemic cells. Complete inhibition by kalafungin at 48 h was observed at 0.1 μg/ml; the 50% inhibition dose (ID_{50}) of kalafungin was 0.018 μg/ml.

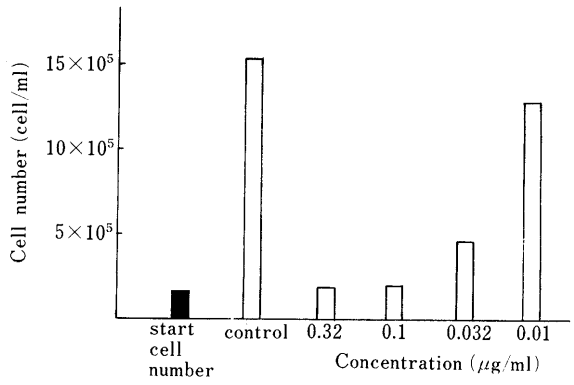


Fig. 1. Effect of Kalafungin on the Growth of L 5178 Y Cell *in Vitro* during Incubation for 48 h

Discussion

It was found that an alkalophilic actinomycete, strain OPC-553 regarded as *Nocardiopsis dassonvillei* subsp. *prasina*[12] produced kalafungin which showed a marked inhibitory activity against L5178Y mouse leukemic cell *in vitro* (Fig. 1). Kalafungin containing the naphthoquinone skeleton in the molecule is the antifungal antibiotic isolated from the culture broth of *Streptomyces tanashiensis* by Bergy *et al.*[3] This antibiotic was also reported by E. L. Rosenfield[13] to show anthelmintic activity. However, the isolation of kelafungin from an alkalophilic actinomycete and its cytotoxicity are reported for the first time in this paper. As shown in Fig. 1, the cytotoxicity of kalafungin was very strong and its 50% inhibition dose (ID_{50}) was 0.018 μg/ml. In addition to kalafungin, there are nanaomycin-D,[14] granaticin,[15] frenolicin,[16] lactoquinomycin-A[17] and -B[18] which contain the naphthoquinone skeleton in the molecule and show cytotoxic activity. Among them, lactoquinomycin-A (Chart 1) is the glycoside of kalafungin. Lactoquinomycin-A has already been reported to have inhibitory activity against doxorubin resistant mouse leukemic L5178Y cells at a concentration higher than 0.08 μg/ml.[17] This antibiotic was also effective against Ehrlich carcinoma in mice.[17] No work has yet been done on the mechanism of action of kalafungin against L5178Y mouse leukemic cell *in vitro*. However, the mechanism of action of lactoquinomycin-A on this cell has already been reported to be due to the depletion of cellular reduced adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide (NADH) by production of a superoxide radical.[19] Considering that kalafungin is the aglycon of lactoquinomycin-A, the mechanism of action of kalafungin on this cell seems to be similar to that of lactoquinomycin-A. The antitumor activity of kalafungin is in progress, together with its mechanisms.

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