Sparoxomycins A1 and A2, New Inducers of the Flat Reversion of NRK Cells Transformed by Temperature Sensitive Rous Sarcoma Virus

I. Taxonomy of the Producing Organism, Fermentation and Biological Activity

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Streptomyces sparsogenes SN-2325 isolated from a soil sample collected in Hokkaido, was found to produce sparoxomycins A1 and A2, new modulators of proliferation of mammalian cells. Sparoxomycins A1 and A2 reverse the morphology of temperature-sensitive mutant Rous sarcoma virus-infected NRK cells (*src*^{ts}-NRK cells) from the transformed phenotype to the normal phenotype at the permissive temperature.

In the course of our screening for new modulators of proliferation of mammalian cells, we have isolated two new members of pyrimidinylpropenamide antibiotic, sparoxomycins A1 and A2 (Fig. 1) from a culture broth of *Streptomyces* sp. SN-2325. It was found that the antibiotics inhibit the cell cycle progression of *src*^{ts}-NRK cells at the boundary of G_1/S phase, and reverse the morphology of the cells from the transformed phenotype to the normal phenotype at the permissive temperature (32°C). In this paper, we report taxonomy of the producing organism, the fermentation, and biological activities of sparoxomycins A1 and A2. Isolation, physico-chemical properties and structure elucidation of these compounds are reported in the previous paper.[1]

Materials and Methods

Taxonomic Studies

Most of the cultural, physiological and biochemical properties of strain SN-2325 were examined using the methods described by SHIRLING and GOTTLIEB.[2] Morphology on yeast-starch agar (containing soluble starch 1%, yeast extract 0.2%, agar 1.5%, pH 7.3) was observed after incubation at 28°C for 10 days. The colors of mycelia and soluble pigments were determined according to the Color Harmony Manual.[3] The morphology of the spore chains was observed with a scanning electron microscope (model S-430, Hitachi Ltd., Tokyo, Japan). A sample for scanning electron micrography was prepared as follows: cutting an agar block, air-drying it, and sputter it with gold under a vacuum. The temperature range for growth was determined on yeast-starch agar by using a temperature gradient incubator (model TN-3; Advantec Toyo, Japan). The type of diaminopimeric acid (A_2pm) in the cell wall was determined by the method of STANEC and ROBERTS.[4]

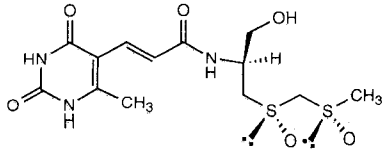
Bacterial Strain

The producing strain SN-2325 was isolated from a soil sample collected in Furano-shi, Hokkaido, Japan. The strain SN-2325 has been deposited in the National Institute of Bioscience and Human-technology, Agency of Industrial Science and Technology, Japan, with the accession No. FERM P-14611.

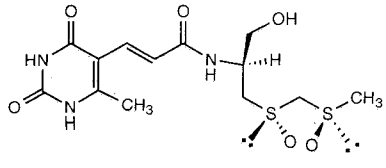
Fermentation

The strain SN-2325 from a mature slant culture was inoculated into two 500-ml cylindrical flasks containing

Fig. 1. Structures of sparoxomycins A1 and A2.



Sparoxomycin A1



Sparoxomycin A2

each 70 ml of an autoclaved medium consisting of glucose 2%, soluble starch 1%, meat extract 0.1%, dry yeast 0.4%, soybean flour 2.5%, NaCl 0.2% and K_2HPO_4 0.005%. The medium was adjusted to pH 7.3 prior to sterilization. The flasks were shaken on a rotary shaker at 300 rpm for five days at 28°C. The resultant seed culture was transferred into two 30-liter jar fermenters charged with 18 liters of the same medium containing 0.1% of CA-123 and 0.025% KM-68 antifoam. The fermenters were agitated at 350 rpm, aerated at 10 liters/minute for 90 hours and the temperature was maintained at 27°C.

Morphological Reversion of *src*^{ts}-NRK Cells

Rat kidney cell line infected with ts25, a T-class mutant of Rous sarcoma virus Prague strain (*src*^{ts}-NRK)[5] was a gift from Dr. UEHARA, NIH Japan, Tokyo. The cells were cultured in EAGLE's minimal essential medium (MEM) supplemented with 10% calf serum (CS, Hyclone Laboratories, Logan, Utah) at permissive temperature (32°C) or at nonpermissive temperature (39°C). The cells (1×10^5 cells/ml) maintained at 32°C were seeded into a 96-well microtiter plate and cultured for two hours at 32°C in 5% CO_2 atmosphere. Solution of various concentration of the antibiotics (5 μl each) was added and morphological reversion of *src*^{ts}-NRK cells were observed under a microscope after 18 to 20 hours incubation at 32°C.

Flowcytometry

Flowcytometry (Coulter, Epics Profile II) was used for analyses of the cell cycle of *src*^{ts}-NRK cells. To analyze the effect of sparoxomycin A2 to the cell cycle progression from G_1 to S, the cells (1.5×10^5 cells/ml) were seeded in 12-well plates and cultured at 39°C for 17 hours. Then, the cells were cultured in the presence or absence of the antibiotics at 32°C for 17 hours. On the other hand the seeded cells (1.5×10^5 cells/ml) were treated with staurosporine (5 ng/ml) and cultured at 32°C for 17 hours for the analysis of the cell cycle progression from G_2 to G_1. After washing with phosphate-buffered saline (PBS), the cells were cultured in the presence or absence of sparoxomycin A2 at 32°C for 4 hours. The distribution of DNA content in individual nuclei was identified by the staining with propidium iodide (sigma). Excitation was done with 488-nm line of an argon laser operating at a continuous output of 150 mW.

Effect of Sparoxomycin A2 on Macromolecular Synthesis

src^{ts}-NRK cells (1.5×10^4 cells/ml) were seeded onto 96-multiwell plate (CELL TITE, SUMIRON) in MEM supplemented with 10% CS and cultured at 32°C for 18 hours. The culture medium was changed to MEM supplemented with 2% CS and [^{3}H]thymidine (2 μCi/ml), [^{3}H]uridine (2 μCi/ml, ICN Radiochemicals) or [^{14}C]-proline (0.1 μCi/ml, ICN Radiochemicals) was added to the cells. The serial dilution of sparoxomycins A2 was added simultaneously. After 4-hour labeling, the cells were harvested and the trichloroacetic acid-insoluble fractions were collected and the radioactivity was counted by using a liquid scintillation counter.

Results and Discussion

Taxonomy

The vegetative mycelium of strain SN-2325 is well developed and branched without fragmentation. The aerial mycelium branched monopodially, and spore chains with relatively tight or compact spirals were formed on the aerial mycelium. Each spore was characterized by its rugose surface (Fig. 2).

The results of cultural characteristics of strain SN-2325

Fig. 2. Scanning electron micrograph of spore chains of strain SN-2325 grown on yeast-starch agar for 10 days.

Bar represents 100 nm.

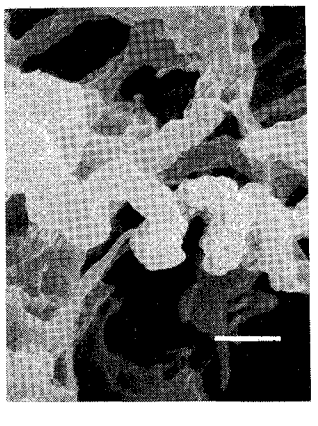


Table 1. Cultural characteristics strain SN-2325 on various media.

	Aerial mycelium	Vegetative mycelium, reverse color	Soluble pigment
Yeast extract-malt extract agar (ISP-2)	Good, ashes (7fe)	Good, cinnamon (3le)	None
Oatmeal agar (ISP-3)	Good, taupe gray (7ih)	Good, dark brown (3nl)	None
Inorganic salts-starch agar (ISP-4)	Good, taupe gray (7ih)	Good, camel (3ie)	None
Glycerol-asparagine agar (ISP-5)	Good, white (a)	Good, beige brown (3ig)	None
Peptone-yeast extract-iron agar (ISP-6)	None	None	None
Tyrosine agar (ISP-7)	Good, white (a)	Good, golden brown (3pg)	None

on various agar media are listed in Table 1. The vegetative mycelia were yellowish brown to brown, and no distinctive pigments were produced. The aerial mycelia were colored with a shade of gray and hygroscopic. Melanoid pigments or other significant diffusible pigments were not produced in any agar medium tested. Utilization of carbon source of strain SN-2325 is summarized in Table 2, and growth occurred at temperature between 12 and 31°C. Analyses of whole cell hydrolysate of strain SN-2325 revealed the presence of major amounts of L,L-A_2pm. According to the classification of LECHEVALIER and LECHEVALIER, the strain has a type I cell wall. On the basis of the morphological and chemical characteristics, it was concluded that strain SN-2325 is classified in the genus *Streptomyces*. As compared with previously described members of this genus[6~10], strain SN-2325 was considered to resemble the following six species: *S. antimycoticus*, *S. cuspidosporus*, *S. hygroscopicus* subsp. *hygroscopicus*, *S. melanosporofaciens*, *S. sparsogenes*, and *S. violaceusniger*. Comparison of carbon utilization among strains tested indicated that SN-2325 was closely related to *S. sparsogenes*. Therefore, we named the strain *S. sparsogenes* SN-2325.

Table 2. Carbon utilization of strain SN-2325.

Carbon source	Growth
L-Arabinose	+
D-Xylose	+
D-Glucose	+
D-Fructose	+
Sucrose	+
i-Inositol	−
L-Rhamnose	+
Raffinose	+
D-Mannitol	+
D-Galactose	+

+; Positive, −; negative.

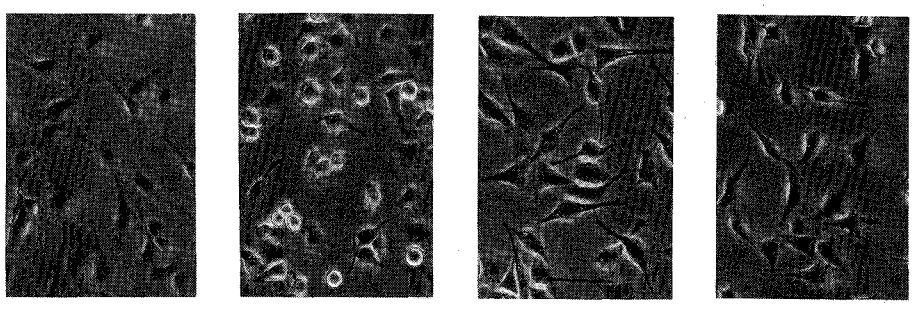
Fermentation

Streptomyces sparsogenes SN-2325 was cultured in a 30-liter jar fermenter containing glucose 2%, soluble starch 1%, meat extract 0.1%, dry yeast 0.4%, soybean flour 2.5%, NaCl 0.2% and K_2HPO_4 0.005%, 0.1% of CA-123 and 0.025% KM-68 antifoam. Production of inducers of flat reversion of *src*^{ts}-NRK cells at the permissive temperature (32°C), growth of the microorganism, and pH of the medium were monitored during the fermentation. The active principles were produced in broth and mycelium, and the activity reached maximum on the 3rd day.

Biological activities

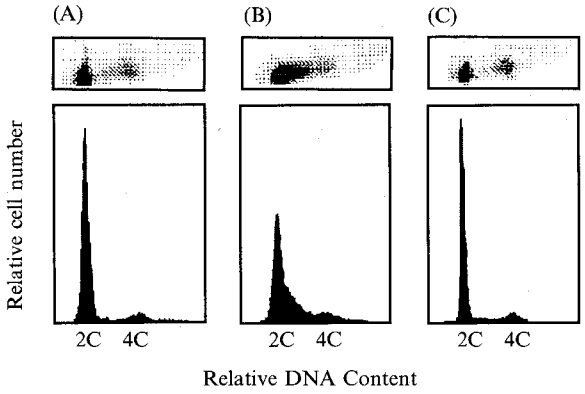
The effect of sparoxomycins A1 and A2 on the morphology of *src*^{ts}-NRK cells are shown in Fig. 3. When *src*^{ts}-NRK cells were grown at the permissive temperature (32°C), many round transformed cells were observed (Fig. 3, B). At the nonpermissive temperature (39°C), the cell growth was suppressed and typical contact inhibition was observed (Fig. 3, A). When sparoxomycins A1 or A2 was added to the cell culture at 32°C, the cell growth was reduced and the transformed morphology was reversed to the normal one after 16 hours (Fig. 3, C and D). The morphology reversion was observed at concentration between 10 and 1000 μg/ml of the antibiotics. Trypan blue staining of the cells indicated that the antibiotics did not show any cytotoxicity within the concentration range (data not shown). When the antibiotics were removed from the culture, the morphology was reversed again to the transformed phenotype. Thus, sparoxomycins A1 and A2 are reversible inducers of the flat reversion activity on *src*^{ts}-NRK cells at 32°C.

Fig. 3. Morphology of *src*^{ts}-NRK cells.



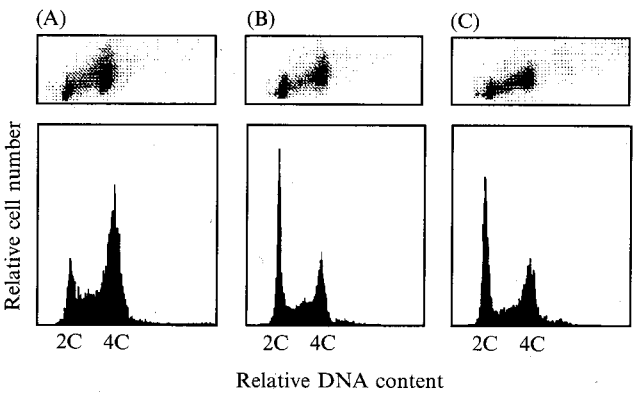
The cells were cultured at 39°C without sparoxomycins (A), cultured at 32°C without sparoxomycins (B), cultured at 32°C with sparoxomycins A1, 100 μg/ml (C), and cultured at 32°C with sparoxomycins A2, 100 μg/ml (D).

Fig. 4. Effects of sparoxomycin A2 on the cell cycle progression of *src*^{ts}-NRK cells synchronized in the G_1 phase.



The *src*^{ts}-NRK cells were synchronized in the G_1 phase (A) by incubation at 39°C for 17 hours. Then, the cells were maintained at 32°C for 17 hours without sparoxomycin A2 (B), or with sparoxomycin A2 (100 μg/ml) (C).

Fig. 5. Effects of sparoxomycin A2 on the cell cycle progression of *src*^{ts}-NRK cells synchronized in the G_2 phase.



The *src*^{ts}-NRK cells were also synchronized in the G_2 phase (A) by treatment with staurosporine (5 ng/ml) at 32°C for 17 hours. Then, the drug was washed out and the cells allowed to progress into mitosis at 32°C for 4 hours in the absence (B) or in the presence of sparoxomycin A2 (100 μg/ml) (C).

Since sparoxomycins showed the flat reversion activity of *src*^{ts}-NRK cells in the wide concentration range, we tested the effect of sparoxomycin A2 on the cell cycle of *src*^{ts}-NRK cells. Effects of the antibiotic on the cell cycle progression were investigated with flowcytometry. The *src*^{ts}-NRK cells cultured at 32°C showed a typical histogram of DNA contents distribution (Fig. 4, B) with peaks at 2C DNA (cells in G_0/G_1 phase) and at 4C DNA (G_2/M phase) as well as a broad band between the two peaks (S phase). The histogram of DNA content of the antibiotic-treated cells (Fig. 4, C) resembled that of the normal cells cultured at 39°C (Fig. 4, A). When the cells treated with staurosporine (5 ng/ml) were released from the G_2 arrest, the pattern of DNA contents of sparoxomycin A2 treated cells (Fig. 5, C) was almost the same as that of no-drug control (Fig. 5, B). These observation suggests that sparoxomycin A2 inhibits selectively the cell cycle progression of *src*^{ts}-NRK cells in G_1 phase at a final concentration of 100 μg/ml. Then the effect of sparoxomycins A2 on macromolecule synthesis was investigated in *src*^{ts}-NRK cells. The antibiotic poorly inhibited protein, RNA and DNA syntheses at 300 μg/ml. Several compounds which reversed the transformed morphology of *src*^{ts}-NRK cells to the normal morphology were already reported *e.g.* herbimycin A[11], reveromycin A[12] and phosmidosine[13]. Since both the effects of sparoxomycins A2 on the activity of p60$^{v\text{-}src}$ kinase and the amount of p60$^{v\text{-}src}$ were also very weak at the dose (data not shown), the molecular target of this compound is different from herbimycin A. It requires a further investigation to clarify the mechanism of action of sparoxomycins.

The effects on the other tumor cell lines were investigated using the major component, sparoxomycin A2. Sparoxomycin A2 showed almost the same MIC (100 μg/ml) against the normal (NIH 3T3) and the transformed cell lines (K-*ras*, *abl*, *fos*), whereas morphology reversion of these transformed cells was not observed at the concentration.

Antimicrobial activities of sparoxomycin A2 were determined by the conventional paper disc method. Sparoxomycin A2 showed weak antimicrobial activities against *Escherichia coli* AB 1157 (bacteriostatic inhibition zone, 9.5 mm) and the following two hypersensitive bacteria, *Escherichia coli* BE 1186 (inhibition zone, 9.2 mm; bacteriostatic inhibition zone, 17.2 mm) and *Pseudomonas aeruginosa* N-10 L-form (bacteriostatic inhibition zone, 11.9 mm) at the dose of 40 μg/disc. However sparoxomycin A2 showed no activity against *Staphylococcus aureus* FDA 209P, *Pyricularia oryzae*, *Botrytis cinerea*, *Candida albicans*, and *Chlorella vulgaris* at the dose.

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References

1) UBUKATA, M.; T.-I. MORITA, M. URAMOTO & H. OSADA: Sparoxomycins A1 and A2, new inducers of the flat reversion of NRK cells transformed by temperature sensitive Rous sarcoma virus. II. Isolation, physicochemical properties and structure elucidation. J. Antibiotics 49: 65~70, 1996
2) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
3) JACOBSON, E.; W. C. GRAUVILLE & C. E. FOGS: Color harmony manual, 4th ed. Container Corporation of America, Chicago, 1958
4) STANECK, J. L. & G. D. ROBERTS: Simplified approach to identification of aerobic actinomycetes by thin-layer chromatography. Appl. Microbial. 28: 226~231, 1974
5) CHEN, Y. C.; M. J. HAYMAN & P. K. VOGT: Properties of mammalian cells transformed by temperature-sensitive mutants of avian sarcoma virus. Cell 11: 513~521, 1977
6) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. II. Species descriptions from first study. Int. J. Syst. Bacteriol. 18: 69~189, 1968
7) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. III. Additional species descriptions from first and second studies. Int. J. Syst. Bacteriol. 18: 279~392, 1968
8) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. IV. Species descriptions from second, third and fourth studies. Int. J. Syst. Bacteriol. 19: 391~512, 1969
9) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type strains of *Streptomyces*. V. Additional species descriptions. Int. J. Syst. Bacteriol. 22: 265~394, 1972
10) PRIDHAM, T. G. & H. D. TRESNER: Family VII. Streptomycetaceae Waksman and Henrici 1943, 339. *In* BERGEY's Manual of Determinative Bacteriology, 8th ed. *Eds.*, E. D. BUCHANAN & N, E. GIBBONS, pp. 747~845, The Williams & Wilkins Co., Baltimore, 1974
11) UEHARA, Y.; H. FUKAZAWA, Y. MURAKAMI & S. MIZUNO: Irreversible inhibition of v-*src* tyrosine kinase activity by herbimycin A and its abrogation by sulfhydryl compounds. Biochem. Biophys. Res. Commun. 162: 801~809, 1989
12) TAKAHASHI, H.; H. OSADA, H. KOSHINO, M. SASAKI, R. ONOSE, M. NAKAKOSHI, M. YOSHIHAMA & K. ISONO: Reveromycins, new inhibitors of eukaryotic cell growth. II. Biological activities. J. Antibiotics 45: 1414~1419, 1992
13) MATSUURA, N.; R. ONOSE & H. OSADA: Morphological reversion activity of phosmidosine and phosmidosine B, a newly isolated derivative, on *src* transformed NRK cells. J. Antibiotics 49: 361~365, 1996