An Antimutagenic Metabolite, Streptovaricin C, Isolated from *Streptomyces* sp.

Kazuhiro OOKA,^a Michiyasu TANAKA,^a Tsutomu ISHIKAWA,^b and Fumio KATO*,^a

Department of Microbiology, School of Pharmaceutical Sciences, Toho University,^a 2-2-1 Miyama, Funabashi, Chiba 274-8510, Japan and Department of Medicinal Organic Chemistry, School of Pharmaceutical Sciences, Chiba University,^b 1-33 Yayoichou, Inage, Chiba, Chiba 263-8522, Japan. Received August 14, 1998; accepted October 5, 1998

***Streptomyces* sp. KM1-30 was isolated from soil as a producer of antimutagens by screening with a modified Ames test. The chemical structure of the antimutagenic metabolite was identified as streptovaricin C, which is known to inhibit DNA dependent RNA polymerase from *E. coli* and RNA dependent DNA polymerase from RNA tumor viruses, by MS and ¹H-, ¹³C-NMR analyses. Addition of streptovaricin C to the cultures of UV treated *Salmonella typhimurium* TA100 or Trp-P-2-treated *S. typhimurium* TA98 decreased the frequency of mutation without a decrease in viable cell counts. The effect of streptovaricin C to the mutation induced by UV and Trp-P-2 was not desmutagenic, but antimutagenic.**

Key words antimutagen; streptovaricin C; mutation; Ames test

There are a number of mutagenic substances in our environment. Recently, mutagens from cooked meat and fish were isolated and identified as pyrolyzates of amino acids such as Trp-P-1, Trp-P-2 and Glu-P-1.^{1–3)} It would be useful to find chemicals which reduce or counteract this mutagenic activity by direct reaction with mutagens, *i.e.* a desmutagen, or by an indirect effect on mutagenesis, *i.e.* an antimutagen. Various natural compounds have been reported as antimutagens.^{4,5)}

We suggested that strains of actinomycetes, by which many bioactive metabolites such as antibiotics and antitumor agents are produced,⁶⁾ synthesize metabolites capable of inhibiting mutagenicity. We screened the productivities of antimutagenic metabolites from strains of Actinomycetes isolated from soils by a modified procedure of the Ames test.⁷⁾ This paper describes the isolation and identification of an antimutagen produced by *Streptomyces* sp. KM1-30 and the antimutagenic activity on various mutagens.

MATERIALS AND METHODS

Chemicals Trp-P-2, 9-aminoacridine, and 4-nitroquinoline-*N*-oxide(4-NQO) were used as mutagens. Other chemicals used were of a special grade.

Microorganisms and Cultivation Strains of actinomycetes were isolated from soil and incubated in a yeast extract–malt extract medium at 27 °C for 4–7 d with vigorous shaking. The culture broth was applied to the antimutagen test. For antimutagen assay, *Salmonella typhimurium* TA98 [*hisD* 3052, *rfa*, Δ (*gal*, *chl*, *uvrB*, *bio*)/pKM101(*mucAB*, Ap^R)] and TA100 [*hisG*46, *rfa*, Δ (*gal*, *chl*, *uvrB*, *bio*)/pKM101 (*mucAB*, Ap^R)]⁸⁾ were used. Those strains were inoculated into 20 ml of Heart infusion (HI) broth (Eiken Kagaku Co.) and incubated at 37 °C for 12–16 h with shaking (240 rpm.).

Antimutagen Test Sterilized paper disks (8 mm in diameter) were dipped into the culture broth and air dried. The filters were then placed onto agar assay plates prepared as follows: 0.1 ml of overnight culture of *S. typhimurium* TA98, 0.1 ml of 9.7 nmol of Trp-P-2 and 0.5 ml of S9 mix were mixed and incubated for 20 min at 37 °C. The mixture was

suspended in 2.5 ml of soft agar supplemented with 50 μ mol of histidine and biotin, then overlaid onto the basal layer made from 20 ml of Minimal glucose agar (MGA). The agar plates were incubated at 37 °C for 48 h. Antimutagenic activity was detected by growth inhibition zones with faint background growth of strain TA98 around the paper disk. When UV was used as a mutagen, cells of overnight cultures of strain TA100 were harvested and washed with 0.1 M phosphate buffer (pH 7.4), then resuspended in the same buffer. One ml of the cell suspension was illuminated by UV (1.8 J/m²) in a petri dish (4.7 cm in diameter), and 0.1 ml of the UV irradiated cell suspension was mixed with the soft agar and overlaid onto MGA plates as described above.

Assay of Antimutagenic Activity Trp-P-2 solution (1.5 ml) was mixed with 7.5 ml of S9 mix and 1.5 ml of overnight cultures of strain TA98, then incubated at 37 °C for 20 min. The cells of TA98 were washed with a phosphate buffer (pH 7.4) and resuspended in 6 ml of the buffer and 1.5 ml of HI broth. Each 0.5 ml aliquots was mixed with 10 μ l of a solution containing various concentrations of antimutagen, and incubated at 37 °C for 20 min. Those treated cells were mixed with soft agar and overlaid onto MGA plates. The number of viable cells was determined by plating the 10⁵ diluted cell suspension on nutrient agar (NA) plates. When UV was used as a mutagen, 2 ml of overnight culture of TA100 was centrifuged and the cells were washed and resuspended in 2 ml of phosphate buffer. The cell suspension exposed to UV (0.1 ml) was mixed with 0.4 ml of HI broth and 10 μ l of a solution containing various concentrations of antimutagen, then the mixture incubated at 37 °C for 20 min. After the incubation, the cell suspension was mixed with 2.5 ml of soft agar and overlaid onto MGA plates. The plates were incubated at 37 °C for 48 h in the dark and colonies on the plates were counted.

Purification and Identification of the Antimutagen *Streptomyces* sp. KM1-30 was cultivated in 3 l of YM medium at 27 °C for 4 d. Culture filtrate adjusted to pH 4 was extracted with ethyl acetate. The organic layer was washed with dist. water and concentrated *in vacuo*. The crude extract (0.18 g) was applied to Sephadex LH-20 column chromatography (i.d. 2.5 cm $\times$ 30 cm) and eluted with *n*-hexane. The ac-

* To whom correspondence should be addressed.

Fig. 1. Assay of Antimutagenic Activity of the Metabolite on the Agar Plates

A: Antimutagenic activity to Trp-P-2 treated cells of *S. typhimurium* TA98, B: antimutagenic activity to UV treated cells of *S. typhimurium* TA100. Conditions for the mutagenic treatments are described in the text. Numbers on paper disks indicate the amount of the metabolite loaded: 1, 0.4 µg/disk; 2, 0.2 µg/disk; 3, 0.1 µg/disk; 4, control.

Table 1. Antimutagenic Effect of Streptovaricin C on Various Mutagens

Mutagen	Conc. of streptovaricin C (µg/ disk)				
	1.5	0.75	0.4	0.2	0.1
UV irradiation (4.7 mJ/plate)	3.0*	2.4	2.0	1.3	±
Trp-P-2 (2.5 µg/plate)	2.7	2.3	1.2	1.2	±
9-Aminoacridine (0.1 mg/plate)	1.9	1.6	1.3	±	±
4-NQO (0.5 µg/plate)	3.0	2.6	2.3	1.6	±

*: indicates the diameter (cm) of the inhibition zone of *his*⁻ to *His*⁺ reversion.

tive fractions were combined and concentrated to dryness. The residue (25.3 mg) was dissolved in methanol and applied to an HPLC Column (TSK-GEL ODS-80TM column, 4.6 mm×150 mm) in methanol-H₂O (40:60, v/v) at a flow rate of 1 ml/min. Finally 6.75 mg of antimutagenic metabolite was obtained.

RESULTS AND DISCUSSION

We have screened antimutagenic activity in the culture broths of about 1000 strains of actinomycetes isolated from soil. Among the strains which produced some antimutagenic metabolites, we selected strain KM1-30 because of its highly stable antimutagenic activity relative to heat and pH.

The antimutagenic metabolite extracted from the culture broth of strain KM1-30 was purified, and its antimutagenic activity to mutation induced by various mutagens was examined. As shown in Fig. 1, the antimutagenic activity of the metabolite was observed as clear zones around paper disks containing various concentrations of the metabolite. Table 1 shows the inhibitory effect of the metabolite on the mutagenesis of *S. typhimurium* TA98 or TA100 induced by UV irradiation (transition and transversion), Trp-P-2 (base substitution and frame shift), 9-aminoacridine (frame shift) and 4-NQO (base substitution). These results, that the antimutagenic activity of the metabolite was not dependent on mutagens, suggested that it acts on a common process in induced mutagenesis resulting in the suppression of *his*⁻ to *His*⁺ reversion.

The structure of the antimutagenic metabolite produced by strain KM1-30 was determined. Physico-chemical properties of the metabolite were as follows: FAB-MS, *m/z* 769; mp 172.9–178 °C; molecular formula, C₄₀H₅₀NO₁₄; UV λ_{max},

Fig. 2. Structure of the Antimutagenic Metabolite of *Streptomyces* sp. KM1-30 and Chemical Shifts of the Carbon Atoms

Figures in the parentheses show chemical shifts of streptovaricin C assigned by Stanley and Rinehart.

244, 260(s), 320(s) and 428 nm. The results of ¹H-, ¹³C-NMR analyses were very consistent with that of an ansamycin antibiotic, streptovaricin C,^{9,10} as shown in Fig. 2. Analysis of the amino acid composition of the cell wall of strain KM1-30 showed the presence of L,L-diaminopimelic acid, glycine and glutamic acid (data not shown) and indicated that strain KM1-30 belongs to the genus *Streptomyces*.¹¹ As streptovaricin C producers, *S. spectabilis* and its variants are known,^{12,13} but the cultural characteristics of strain KM1-30 were different from those of *S. spectabilis*¹⁴ (Table 2). Therefore, *Streptomyces* sp. KM1-30 may be a newly identified producer of the ansamycin antibiotic, streptovaricin C.

Although streptovaricin C is well known as an antimicrobial and antiviral metabolite which inhibits DNA dependent RNA polymerase and RNA dependent DNA polymerase,^{15–17} there are no reports on the antimutagenic activity of streptovaricin C. We investigated in greater detail the effect of streptovaricin C on the repression of *his*⁻ to *His*⁺ reversion induced by UV or Trp-P-2 in *S. typhimurium* TA100 or TA98. As shown in Figs. 3a and 3b, the addition of streptovaricin C to cultures of TA100 or TA98, which were treated with UV irradiation or Trp-P-2, decreased the reversion fre-

Table 2. Comparison of Cultural Characteristics of Strain KM1-30 with That of *Streptomyces spectabilis* on Various Media

	KM1-30	<i>S. spectabilis</i>
Nutrient starch agar		
Substrate mycelium	Cream	Cream turning to orange
Aerial mycelium	None	Deep orange, paler sectors
Reverse side color	Cream	Yellow
Hydrolysis	Negative	Positive
Waksman's starch agar		
Substrate mycelium	Cream to gray	Cream color flecked with orange
Aerial mycelium	Pale moss-green	Pale orange
Hydrolysis	Negative	Positive
Bennett's agar		
Substrate mycelium	Brown turning to gray	Yellow turning to orange red
Aerial mycelium	Pale gray	Trace of orange red to gray
Reverse side color	Gray	Yellow
Utilization of:		
D-Glucose	+	+
D-Xylose	+	+
L-Arabinose	+	—
L-Rhamnose	+	—
D-Fructose	+	+
D-Galactose	+	+
D-Mannitol	+	+
myo-Inositol	+	+
Sucrose	+	+

quencies of those cells, although there was no effect on the viability of strains TA98 and TA100 in the reaction mixtures containing various concentrations of streptovaricin C. At the same concentration of streptovaricin C, the decrease in the numbers of UV-induced revertants were greater than the decreases which occurred for Trp-P-2 induced reversions. In each case, reduction in the reversion frequency of UV-induced TA100 and Trp-P2-induced TA98 to 1/10 was attained by the addition of streptovaricin C at concentrations of 3.125 or 12.25 $\mu\text{g/ml}$, respectively. The minimum inhibitory concentration (MIC) of streptovaricin C for the strains of TA100 and TA 98 were 12.5 $\mu\text{g/ml}$ or 25.0 $\mu\text{g/ml}$, respectively. These results suggest that streptovaricin C inhibited the error-prone repair system specifically, by an unknown mechanism or the partial inhibition of RNA polymerase activity by streptovaricin C, resulting in a decrease in transcription including that of the genes for an error-prone repair system.

We investigated the effect of rifampicin,¹⁸⁾ a well known RNA polymerase inhibitor, on UV-induced mutagenesis. As shown in Fig. 4, only a slight decrease in the reversion frequency was observed by the addition of rifampicin. This result suggests that the partial inhibition of RNA polymerase by the inhibitor did not affect mutagenesis. Although we need to elucidate the antimutagenic mechanism of streptovaricin C, this activity of streptovaricin C is a novel type of bioactivity for the compound.

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Fig. 3. Inhibitory Effect of Streptovaricin C on the Mutagenesis Induced by UV Irradiation or by Trp-P-2 Treatment in *S. typhimurium* TA98 or TA100

a: inhibitory effect on the UV-induced mutation in *S. typhimurium* TA100. b: inhibitory effect on Trp-P-2-induced mutation in *S. typhimurium* TA98. Mutation frequencies are expressed as the number of *His*⁺ colonies/number of viable cells. Spontaneous reversion frequencies of TA 98 and TA 100 were 2.2×10^{-5} and 4.08×10^{-7} , respectively.

Fig. 4. Inhibitory Effect of Rifampicin on the Mutagenesis Induced by UV Irradiation in *S. typhimurium* TA100

As described in the text, strain TA100 was irradiated with UV and the cell suspension (0.1 ml) was mixed with 0.4 ml of HI broth and 10 μl of a solution containing various concentrations of rifampicin, then incubated at 37 $^{\circ}\text{C}$ for 20 min. After the incubation, the cell suspension was mixed with 2.5 ml of soft agar and overlaid onto MGA plates. Mutation frequencies are expressed as the number of *His*⁺ colonies/number of viable cells. The spontaneous reversion frequency of strain TA100 was 4.08×10^{-7} .

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