NOTES

AN IMPROVED SCREENING METHOD FOR ANTIPHAGE ANTIBIOTICS AND ISOLATION OF SARKOMYCIN AND ITS RELATIVES

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Hitherto, several screening procedures[1,2] for compounds showing inhibitorial action on the multiplication of DNA phages of λ and T series have been developed. In contrast very little effort has been directed toward finding inhibitors of RNA phages.

In this paper, we wish to report a simple and convenient method developed by the authors for the assay of fermentation broths for activity against RNA phage and application of the method leading to isolation and identification of sarkomycin and dihydrosarkomycin.

The method is based on the use of the well-characterized RNA phage f_2[3] with *Escherichia coli* K12 W 2252 as host organism.

Screening Procedure

The combination of RNA phage f_2 and *E. coli* K12 W 2252 (Hfr, met$^-$, λ^-) was used throughout this screening system. The antiphage activity was determined by an agar plate method employing double layers in Petri dishes.

Medium of the following composition, with or without agar, was used for the cultivation of *E. coli* K12: Polypeptone 1 %, yeast extract 0.1 %, glucose 0.1 %, NaCl 0.1 %, $CaCl_2$ 0.05 % and agar 1 % where necessary. The pH of the medium was adjusted to 7.2 before sterilization.

A stock of phage f_2 was prepared as follows: Fifteen ml of the above liquid medium in a test tube (2×20 cm) were inoculated with approximately 3×10^7 cells per ml of *E. coli* K12 and shake-cultured for 2 hours at 37°C. Phage f_2($10^3\sim10^4$ particles per ml) was then introduced into the cultured broth. After about 5 hours, lysis of the host organism occurred and the culture became transparent. A few drops of chloroform were added to the lysate in order to complete the lysis. The lysate was successively centrifuged (10,000 rpm for 10 minutes) to remove cell debris and the supernatant was used as a stock preparation. The phage titer of this stock was $1.2\sim1.4\times10^{12}$/ml. A buffer solution of 0.002 M KH_2PO_4, 0.008 M Na_2HPO_4 and 0.15 M NaCl (pH 7.4) was used to dilute the phage stock by the factor of 4×10^3.

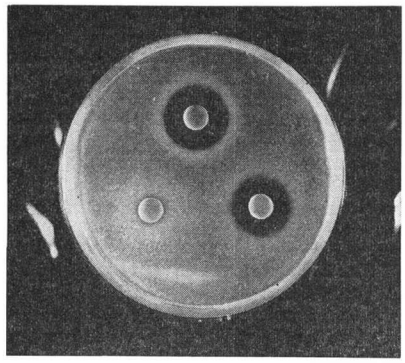
Assay plates were prepared as follows: 14 ml of the agar medium described above was poured into a Petri dish (9 cm in diameter) as the basal layer and after solidification, 2.5 ml of the same medium (containing 0.5 % agar) mixed with a suspension (0.1 ml) of *E. coli* K12 cells obtained by culturing overnight in liquid medium. A suspension of f_2 phage (0.1 ml) was plated on the upper layer. For best results the ratio of f_2 phage to the host bacterium was adjusted to 1:10.

The bioassay of antiphage compounds was conducted by a procedure utilizing paper disks impregnated with test solutions. After the impregnated disks were placed on the agar surface the plates were allowed to stand overnight in a refrigerator at 7°C in order to facilitate diffusion of the active substance into the medium. The plates were then incubated at 37°C for 6.5 hours.

As shown in Fig. 1, the antiphage activities of rifampicin and sarkomycin[4] can be discerned clearly by the growth zones of the host bacterium around the paper disks. When the test compound possesses both antiphage activity and antibacterial activity, an inhibitory zone appears on the inner-side and a stimulatory growth zone at the outer-side. Rifampicin,[5] an antibiotic possessing both antibacterial and antiphage activities, shows these typical double zones as illustrated in Fig. 1.

Fig. 1. Effects of rifampicin and sarkomycin on phage f_2.

Top: soaked with rifampicin at the level of 25 mcg/disk, lower left; sarkomycin 10 mcg/disk, and lower right; sarkomycin 30 mcg/disk. Sarkomycin was less effective against *E. coli* K12 W 2252 at lower concentration, but showed distinct antiphage effect in stimulating the growth zone of host cells. (lower left)



Isolation and Characterization of Sarkomycin and its Relatives

Of 560 *Streptomyces* strains tested by the above screening procedure, the strain designated *Streptomyces* sp. 9948 N_1 produced a culture broth possessing definite antiphage activity. When the producing organism was cultured in a medium containing starch 1 %, glycerol 1 %, glucose 0.5 %, meat extract 0.5 %, yeast extract 0.2 %, Polypeptone 0.3 %, casein 0.1 %, $CaCO_3$ 0.2 % and thiamine hydrochloride 10 mg/liter (pH 7.2 before sterilization) at 27°C for 32 hours in a jar fermentor, maximal production of the inhibitory agent was observed.

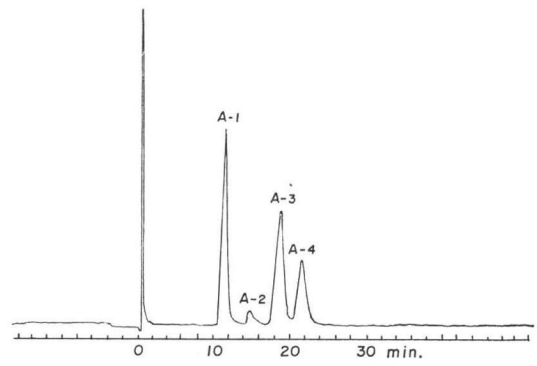
For isolation of the active principle the broth was filtered and the filtrate (10 liters) treated with 0.5 % of activated carbon to remove impurities. The filtrate was extracted with *n*-butanol (3 liters) at pH 3.0. Almost all of the activity was extracted into *n*-butanol and was then transferred into an aqueous phase by treatment with 0.2 N sodium bicarbonate solution (500 ml) while cooling with ice. After adjusting the aqueous solution to pH 3.0, the active principle was extracted continuously with methylene chloride, and the extract dried over sodium sulfate. Upon removal of the methylene chloride under reduced pressure, a yellowish oil (5 g) was obtained, which was unstable and tended to polymerize when exposed to air. The oily extract contained at least two active spots, that were detectable by TLC on silica-gel plate (Kieselgel G, solvent : chloroform - methanol - acetone, 16:1:2) followed by bioautography using *Xanthomonas oryzae*.* Because of the apparent acidic nature of the active principle, methylation of free carboxyl groups was attempted at an earlier stage of the purification procedure to prevent polymerization. Consequently, the methylene chloride extract was evaporated to dryness and the residue dissolved in methanol (100 ml) was treated with a large excess of ethereal diazomethane. Although the antibacterial and antiphage activities were decreased by the methylation procedure, the oily substance obtained after removal of the organic solvent was relatively stable. This oily substance was then subjected to separation and identification by GLC and GC-MS procedures.

On gas chromatography, the methylated oil gave four peaks numbered A_1 to A_4 in the order of increasing retention time as shown in Fig. 2.

Further characterization by GC-MS revealed the molecular ion of each component with

Fig. 2. GLC of methylated products.

GLC was carried out using a Shimazu Gas Chromatograph GC-4B PTF equipped with a dual flame ionization detector and developing with nitrogen at a flow rate of *ca.* 50 ml/min. The column was of a 0.4×120 cm (U-shaped) glass tube, packed with 3 % ethylene glycol succinate polyester on chromosorb-w (60~80 mesh).



* *Xanthomonas oryzae* was used as test organism in bioautography since it was more sensitive to the active principle than *E. coli* K12 W 2252.

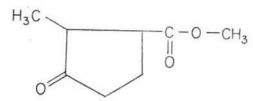
Table 1. Main peaks of components A_1, A_3 and A_4 on mass spectra.

A_1	M^+(156), 141(M-CH_3), 128(M-CO) 125(M-OCH_3), 97(M-$COOCH_3$) 69(base, M-CO-$COOCH_3$)
A_3	M^+(154), 139(M-CH_3), 126(M-CO) 123(M-OCH_3), 95(M-$COOCH_3$) 67(M-CO-$COOCH_3$)
A_4	M^+(168), 140(M-CO), 137(M-OCH_3) 109(M-$COOCH_3$)

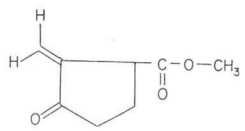
the exception of A_2. The mass spectral data of A_1, A_3 and A_4 are summarized in Table 1. The A_2 peak was too small to allow further characterization.

Separation of the methylated oil by preparative GLC provided reasonable quantities of A_1, A_3 and A_4 in pure form.

The molecular formula $C_8H_{12}O_3$ of A_1 was determined by high resolution mass spectrometry (M^+, calculated, 156.0786; observed, 156.0784). The NMR spectrum of A_1 in $CDCl_3$ showed the signals of a methyl group at δ 1.15 (3H, d), a carbomethoxy group at δ 3.75 (3H, s) and a broad peak at δ 1.8~2.8 (6H, m).



Dihydrosarkomycin methyl ester (I)



Sarkomycin methyl ester (II)

A_1 was identified as dihydrosarkomycin methyl ester (**I**), isolated independently by SUZUKI *et al.** by comparison of the NMR spectra.

A_3 was isolated as an oil of $C_8H_{10}O_3$(M^+, 154). The NMR spectrum in $CDCl_3$ showed the signals of a carbomethoxy group at δ 3.75 (3H, s), two olefinic protons assignable to an exocyclic methylene group at δ 6.16 (1H, d, J=2.7 Hz) and δ 5.58 (1H, d, J=2.4 Hz), four methylenic protons at δ 2.33 (4H, m) and a methine proton overlapped into the methoxy group at *ca.* δ 3.75.

On the basis of NMR spectral data, A_3 was identified as sarkomycin methyl ester (**II**). Sarkomycin methyl ester demonstrated antiphage activity at the concentration of about 160 mcg/ml but dihydrosarkomycin methyl ester was almost inactive at the level of 1 mg/ml.

A_4 was an oil with the molecular formula $C_9H_{12}O_3$ (M^+, *m/e* 168) which was a minor component and almost biologically inactive and was not characterized further.

* Private communication from Dr. S. SUZUKI of the Institute of Physical and Chemical Research.

Summary

1. A simple and convenient method for the assay of antiphage activity of the fermentation broths has been developed with the combination of RNA phage f_2 and *E. coli* K12 W 2252 (Hfr, met$^-$, λ^-).

2. As a result of 560 *Streptomyces* strains being tested by the above screening procedure, the strain designated *Streptomyces* sp. 9948 N_1 produced two antiphage substances which were characterized as sarkomycin and dihydrosarkomycin based on the spectral evidences of NMR and GC-MS.

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