

## A NEW MACROMOLECULAR ANTITUMOR ANTIBIOTIC, C-1027

## II. ISOLATION AND PHYSICO-CHEMICAL PROPERTIES

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A new macromolecular antibiotic C-1027 was obtained from the broth filtrate of *Streptomyces globisporus* C-1027 by precipitation with ammonium sulfate, DEAE-cellulose column chromatography and gel filtration chromatography on a Sephadex G-75 column. This antibiotic, prepared as a white powder, is an acidic polypeptide having an isoelectric point of pH 3.5~3.7 and a molecular weight of 15,000 as determined by SDS-polyacrylamide gel electrophoresis and gel filtration chromatography. The acid hydrolysate of the purified antibiotic C-1027 contained no methionine or tryptophan. From the physico-chemical data, it may be considered to possess a very labile non-protein chromophore.

In the course of our screening for an antitumor antibiotic, antibiotic C-1027 was found employing spermatogonial assay<sup>1)</sup> in the broth filtrate from *Streptomyces globisporus* C-1027 and showed a potent cytotoxic activity against KB carcinoma cells in a tissue culture. This antibiotic exhibited activity against Gram-positive bacteria and prolongs the life span of mice bearing P388 leukemia. A taxonomic study of this antibiotic-producing strain and its fermentation and biological properties have been reported in a previous paper<sup>2)</sup>.

The present paper describes the isolation, purification and physico-chemical properties of antibiotic C-1027.

## Fermentation and Isolation

The procedures used in this study were essentially the same as those employed for the production of antibiotic C-1027, as reported previously<sup>2)</sup>.

The progress of fermentation and isolation was monitored by antimicrobial activity using *Micrococcus luteus* ATCC 9341 as a test organism and antitumor activity by cytotoxicity against KB carcinoma cells in a tissue culture. The eluates following column chromatography were monitored by measuring protein concentration by the method of LOWRY *et al.*<sup>3)</sup>.

The culture broth was centrifuged and filtered with filter paper to remove the mycelial cake. The pH of the culture broth was adjusted to 4 with dilute HCl followed by centrifugation at 2,800 rpm for 15 minutes. To the supernatant (6.35 liters), 3.5 kg of ammonium sulfate were added to precipitate the antibiotic, and the mixture was stirred for 4 hours at 5°C. The precipitate containing the antibiotic was collected by centrifugation at 3,000 rpm for 30 minutes and dissolved in cold distilled water. After centrifugation to remove impurities, the resulting solution was dialyzed overnight in a cellophane tube against ice-cold water with frequent changes. From 6 liters of culture broth, approximately 5 g of protein fraction were obtained by lyophilization. This crude material (2.9 g) was applied onto a

DEAE-cellulose column (OH-form,  $18 \times 3.2$  cm), which was washed with water and eluted with 0.1 M sodium chloride. Active fractions corresponding to antibiotic C-1027 were dialyzed against ice-cold water for 4 hours and lyophilized, giving 355 mg of protein fraction. The active fraction was applied onto a Sephadex G-50 column ( $43 \times 2.2$  cm) and eluted with water. The active fractions were combined, lyophilized and chromatographed on Sephadex G-75 ( $85 \times 1.5$  cm). Distilled water was used to elute the antibiotic and 74 mg of a white powder were obtained.

As mentioned in the subsequent section, the purified sample of antibiotic C-1027 gave a single band staining with 0.1% coomassie brilliant blue on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and a single peak on gel filtration chromatography (GFC), indicating its homogeneity.

It was found that *S. globisporus* C-1027 co-produced a related protein which suppressed the inhibitory activity of antibiotic C-1027 against *M. luteus*. This protein could be separated from antibiotic C-1027 by DEAE-cellulose column chromatography. The details of this antagonistic protein will be described in our next paper.

#### Physico-chemical Properties

Antibiotic C-1027 was obtained as a lyophilized white powder. It was found to be an acidic protein with an isoelectric point of pH 3.5~3.7 as determined by isoelectric focusing on a 5% polyacrylamide gel, provided with 2% Ampholine, pH 2.5~4.5, according to the method of OWEN *et al.*<sup>4)</sup>. It did not show a definite melting or decomposition point and carbonized at about 260°C. It was soluble in water but not in organic solvents such as methanol or acetone.

Fig. 1. UV absorption spectra of antibiotic C-1027.

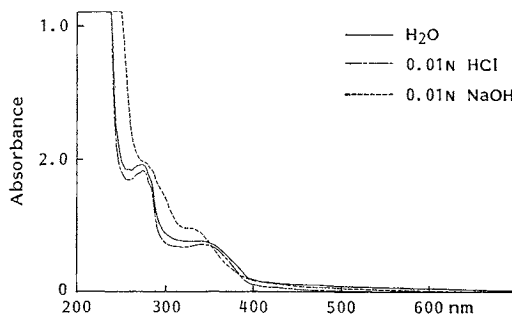


Fig. 2. IR absorption spectrum of antibiotic C-1027 (in KBr).

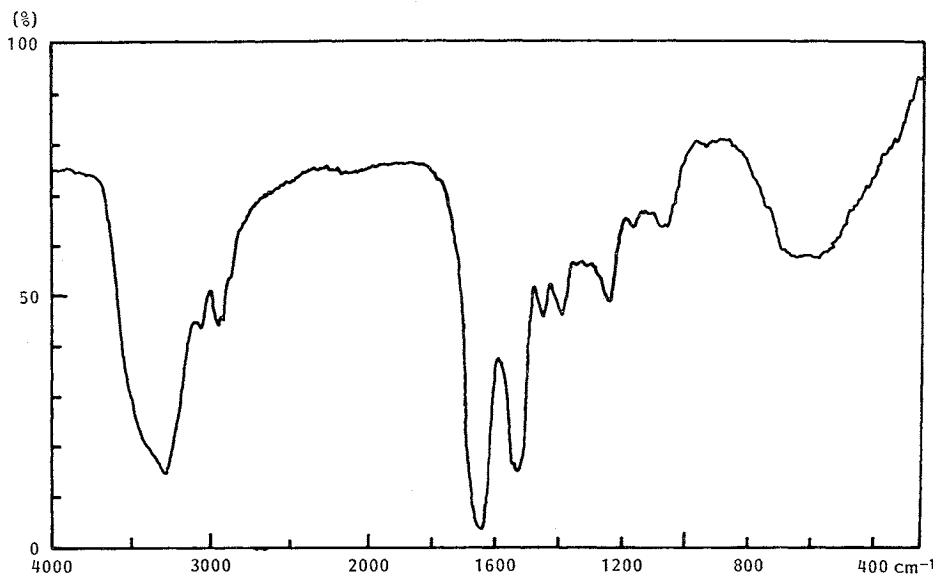


Fig. 3. Calibration curve for determination of the molecular weight of the purified antibiotic C-1027 by gel filtration chromatography (GFC).

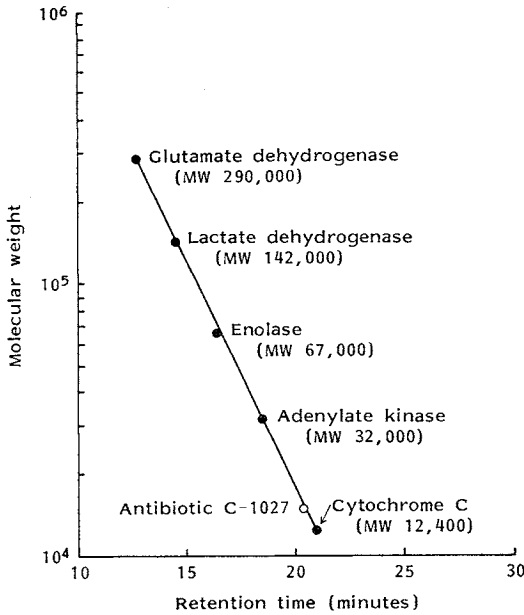
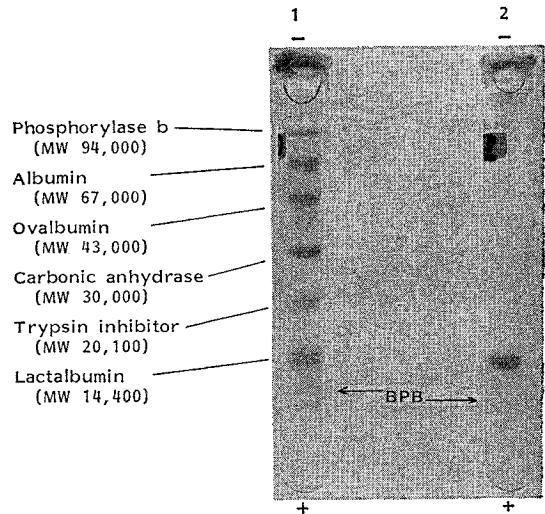


Fig. 4. SDS-polyacrylamide gel electrophoresis of the purified antibiotic C-1027.

Lane 1: Marker proteins, lane 2: purified C-1027.

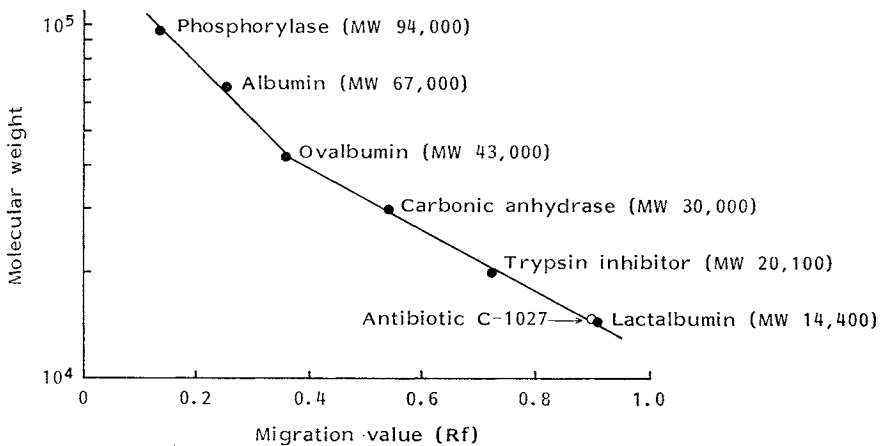


Electrophoresis in 12.5% polyacrylamide gel in 0.05 M phosphate buffer, pH 7.0, containing 0.1% SDS was carried out at 6 mA/tube in the presence of 2-mercaptoethanol.

The gels were stained with 0.1% coomassie brilliant blue R-250 and destained by diffusion in a mixture of methanol - acetic acid - water.

Fig. 5. Calibration curve for determination of the molecular weight of antibiotic C-1027 by SDS-polyacrylamide gel electrophoresis.

Conditions for electrophoresis were as described in Fig. 4.



It gave positive reactions with Folin-Lowry, biuret and ninhydrin, but negative reactions with anthrone and aniline-phthalic acid.

The UV spectrum of antibiotic C-1027 measured in water showed maximum absorption at 275 nm and a definite absorption shoulder between 340 and 360 nm (Fig. 1). Typical peptide bands were evident in the IR spectrum in a KBr tablet (Fig. 2).

The molecular weight of antibiotic C-1027 was determined to be 15,000 by the following gel filtration chromatography using glutamate dehydrogenase, lactate dehydrogenase, enolase, adenylate kinase and cytochrome C as the reference standards: Column, TSKgel G2000SW (600×7.5 mm, Tosoh); mobile phase, 1/15 M phosphate buffer (pH 7.0) containing 0.2 M sodium chloride; flow rate, 1.0 ml/minute; detector, UV at 210 nm or 280 nm. The results are shown in Fig. 3. Furthermore, by the method of WEBER and OSBORN<sup>5)</sup> in the presence of 2-mercaptoethanol, molecular weight was measured on a 12.5%-polyacrylamide gel containing 0.1% SDS for comparison with reference compounds. The results are given in the Fig. 4. Molecular weight markers used were phosphorylase b, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and lactalbumin. The molecular weight was also estimated to be 15,000 (Fig. 5).

The amino acid composition of antibiotic C-1027 was determined by the HPLC using *o*-phthal-

aldehyde as the fluorescence reagent after acid hydrolysis with 6 N HCl at 110°C for 20 hours. In addition, cysteine was measured as *S*-sulfo-cysteine<sup>6)</sup> and tryptophan was determined following hydrolysis with *p*-toluenesulfonic acid and sodium hydroxide<sup>7)</sup>. As shown in Table 1, it consisted of sixteen different amino acids with methionine and tryptophan absent.

The *N*-terminal amino acid was determined

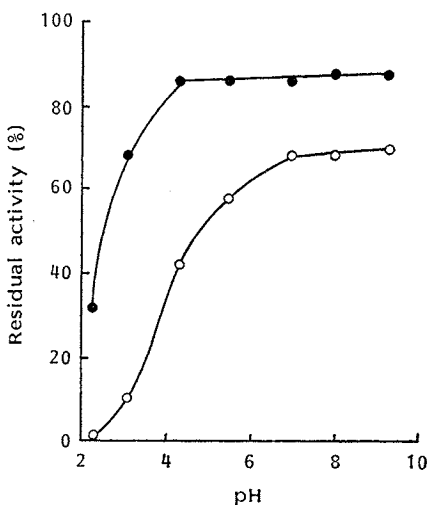
Table 1. Amino acid composition of antibiotic C-1027.

| Amino acid | Content (%) | Amino acid | Content (%) |
|------------|-------------|------------|-------------|
| Asp        | 6.16        | Thr        | 6.27        |
| Ser        | 8.97        | Glu        | 4.74        |
| Pro        | 4.96        | Gly        | 6.41        |
| Ala        | 9.36        | Cys        | 1.90        |
| Val        | 6.85        | Met        | nd          |
| Ile        | 0.76        | Leu        | 3.76        |
| Trp        | nd          | Tyr        | 2.76        |
| Phe        | 4.78        | Lys        | 1.10        |
| His        | 1.19        | Arg        | 1.14        |

nd: Not detected.

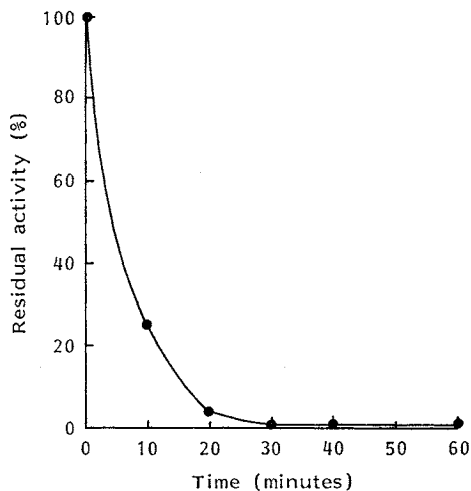
Fig. 6. Effects of pH on antibiotic C-1027 stability.

● 37°C, ○ 60°C.



Residual activity was determined by agar well diffusion method against *Micrococcus luteus* ATCC 9341.

Fig. 7. Effects of UV-irradiation on antibiotic C-1027 stability.



Three mg of antibiotic C-1027 dissolved in 3 ml of water in a Petri dish (3.5 cm in diameter) were irradiated with UV-light (Toshiba, GL-15) from a distance of 25 cm.

The residual activity was determined by agar well diffusion method against *Micrococcus luteus* ATCC 9341.

Table 2. Time course of cytotoxicity of antibiotic C-1027 against KB cells during period of storage.

| Drug              | Condition | Cytotoxicity (ED <sub>50</sub> , µg/ml) |        |         |         |         |
|-------------------|-----------|---|--------|---------|---------|---------|
|                   |           | 0 day                                   | 8 days | 14 days | 21 days | 28 days |
| Antibiotic C-1027 | A         | 0.001                                   | 0.002  | 0.005   | —       | 0.006   |
| Antibiotic C-1027 | B         | 0.001                                   | 0.05   | 0.4     | 1       | >3      |
| Doxorubicin       | A         | 0.01                                    | 0.01   | 0.01    | 0.01    | 0.01    |

Antibiotic C-1027 was dissolved in distilled water at a concentration of 1 mg/ml and stored at 4°C in the dark (A) and at room temperature (B) until use for assay. KB cells ( $2 \times 10^4$  cells/ml) were cultured in EAGLE's minimal essential medium supplemented with 10% calf serum at 37°C under a 5% CO<sub>2</sub>-air atmosphere. After incubation for 24 hours, the antibiotic was added to the culture medium and the incubation was continued for 3 days. Cell growth was determined using a hemocytometer to count viable cells stained with trypan blue. Cytotoxicity was expressed as ED<sub>50</sub> of control growth.

by the DNS method of GRAY and HARTREY<sup>8)</sup> in the presence of SDS and by the DNP method of SANGER<sup>9)</sup>. DNS- and DNP-amino acids after acid hydrolysis of reaction products with 6 N HCl at 105°C for 16 hours were identified by HPLC and TLC. The *N*-terminal amino acid of antibiotic C-1027 was identified as alanine by both methods.

To determine the stability of C-1027 under various conditions, the antibiotic was dissolved in water, adjusted to a specified pH and kept at 37 or 60°C for 1 hour. After the reaction, the solution was placed in a dark room at 5°C, and residual activity was determined by agar well diffusion method against *M. luteus*. As shown in Fig. 6, antibiotic C-1027 was relatively stable in the pH range of 4 to 7, but lost this activity rapidly in strong acid at relatively high temperature. Furthermore, on standing at room temperature, an aqueous solution of the antibiotic gradually lost its cytotoxic activity against KB cells and was rapidly inactivated by UV-light irradiation. These results are shown in Table 2 and Fig. 7.

### Discussion

Many macromolecular antitumor antibiotics have been reported. Several of those are antitumor chromoproteins, neocarzinostatin<sup>10)</sup>, auromomycin<sup>11)</sup>, macromomycin<sup>12)</sup>, actinoxanthin<sup>13)</sup> and sporamycin<sup>14)</sup> which possess a non-protein chromophore. Sporamycin is reported to be a basic antitumor polypeptide, whereas antibiotic C-1027 is an acidic substance based on isoelectric focusing. Neocarzinostatin, macromomycin, auromomycin and actinoxanthin belong to acidic antitumor substances, but they differ from antibiotic C-1027 in physico-chemical properties such as molecular weight, UV spectrum and isoelectric point, and in biological properties<sup>2)</sup>. Thus, to our knowledge, there is no known substance identical with antibiotic C-1027.

The UV spectra of antibiotic C-1027 (Fig. 1) showed a definite absorption shoulder between 340 and 360 nm with the characteristic polypeptide absorption at 275 nm. It suggests that a chromophore is present in the C-1027 molecule having a maximum UV absorption at 340~360 nm, as reported for another protein antibiotic, neocarzinostatin. After the UV-light irradiation for an hour, the antibiotic lost all its activity (Fig. 7) and no absorption shoulder between 340 and 360 nm is observed.

For preparation of the chromophore fraction, purified antibiotic C-1027 was suspended in methanol at a concentration of 1 mg/ml, stirred for 4 hours at -20°C in the dark room and after centrifugation, the supernatant was concentrated *in vacuo*. The residue having antimicrobial activity was analyzed by reversed-phase HPLC with acetonitrile - 20 mM phosphate buffer (pH 7.0) as the mobile phase, monitored at 350 nm, as was also done for the chromophore of auromomycin<sup>15)</sup>. In addition, the protein fraction precipitated by centrifugation showed only UV absorption at 275 nm. From these results, though the chromophore substance has yet to be isolated from antibiotic C-1027, it appears

essential to the expression of the biological activity of the antibiotic as also noted in the case of neocarzinostatin, auromomycin and macromomycin. The isolation, purification and biological property of the non-protein chromophore will be dealt with in the following paper.

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