Note

Isolation of 2'-Deoxycoformycin and Cordycepin from Wheat Bran Culture of *Aspergillus nidulans* Y 176-2*

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Adenosine related antibiotics, such as cordycepin (3'-deoxyadenosine), formycin and 9- β -D-arabinofuranosyl adenine (ara-A), have been reported to show several interesting biological activities, *e.g.* antibacterial, antiviral and antitumor activities. However, these antibiotics are easily catabolized by adenosine deaminase to form corresponding inosine related compounds whose activities are relatively low. As adenosine deaminase occurs extensively in animal body, the *in vivo* activity of these antibiotics is generally not so marked as expected from *in vitro* studies. To overcome this disadvantage, there have been many attempts to synthesize the deaminase-resistant derivatives of these antibiotics,¹⁾ or to use them in combination with an adenosine deaminase inhibitor.^{2,3)} Recently a potent adenosine deaminase inhibitor, 2'-deoxycoformycin (2'-dCF), was isolated from culture of *Streptomyces antibioticus* together with ara-A,⁴⁾ and has been demonstrated to potentiate the biological activities of ara-A or other analogs of adenine nucleosides including cordycepin.^{5,6)}

We found that a newly isolated strain Y176-2 belonging to *Aspergillus nidulans* produced simultaneously 2'-dCF and cordycepin when cultured on wheat bran.

Morphological properties of the newly isolated strain Y176-2 were observed by using an optical microscope and a scanning electron microscope. The shapes of conidial heads were columnar to radiate (40~60 $\mu \times$ 30~60 μ) and conidiophores were smooth-walled. Vesicles were flask-shaped and sterigmata were two series. Conidia were globose, smooth-surfaced and green (2.5~3 μ in diameter). Cleistothecia, huell cells

and asci were observed. Ascospore was purple-red, smooth-walled and lenticular (3~3.5 $\mu \times$ 4.5 μ) with two equatorial and entire margin crests. According to the descriptions by Raper and Fennell,⁷⁾ these morphological properties of strain Y176-2 were closely related to those of *Aspergillus nidulans* group. Therefore, we named strain Y176-2 as *Aspergillus nidulans* Y176-2.

Cytotoxic activity was determined by growth-inhibitory activity against mouse leukemia L5178Y cells in culture. To 9 volumes of the exponentially growing culture of L5178Y cells, 1 volume of sample solution was added. After incubation at 37°C for 48 hr, the number of cells was counted and the dilution rate or the concentration of sample causing 50% growth inhibition was determined (ID₅₀).

Inhibitory activity against fungal adenosine deaminase (obtained from Amano Seiyaku, Co., Ltd.) was assayed as follows: A reaction mixture containing 1.5 mg of adenosine dissolved in 1 ml of 0.1 M sodium citrate buffer, pH 5.6, 0.1 ml of adenosine deaminase solution (1.0 mg/ml) and 0.4 ml of a sample solution diluted with the same buffer was incubated at 45°C. After 30 min incubation, 0.2 ml of the reaction mixture was diluted to 10 ml with 0.1 N HCl and OD₂₈₅ of the diluted solution was measured, which decreases with the progress of deamination. The adenosine deaminase inhibitor activity (unit/ml) is expressed as the reciprocal of the dilution causing 50% inhibition of the deamination.

Ten kg of wheat bran was humidified with 7 liters of deionized water and sterilized at 119°C for 30 min. The sterilized wheat bran was inoculated with the seed

FIG. 1. Effect of the Culture Extract of Strain Y176-2 on Growth of L5178Y Cells.

Cells were cultured in RPMI 1640 medium supplemented with 10% calf serum in the absence of the extract (A) and in the presence of the extract diluted 1:4000 (B), 1:3000 (C), 1:2000 (D), 1:1000 (E), 1:500 (F), and 1:250 (G).

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culture of *A. nidulans* Y 176-2 and incubated for 14 days at 28°C. At the end of incubation period, the culture was soaked in 75 liters of distilled water, stirred for 1 hr at 28°C and filtered to get "culture extract." The culture extract inhibited markedly the growth of L5178Y cells. As shown in Fig. 1, complete inhibition and 50% inhibition were observed by addition of the culture extract diluted 1:250 and 1:3000, respectively. The culture extract also inhibited adenosine deaminase activity (40 units/ml). Both inhibitory activities were easily separated from each other.

FIG. 2. Effect of pH 3-treatment on the Inhibitory Activity of the Culture Extract against L5178Y Cells (○) and that against Adenosine Deaminase (●).

The culture extract was adjusted to pH 3, and treated at 45°C for the periods indicated. The treated extract was adjusted to pH 5.6, and its inhibitory activities against L5178Y cells and adenosine deaminase were measured.

As shown in Fig. 2, adenosine deaminase-inhibitory activity of the culture extract decreased markedly during pH 3-treatment at 45°C, in parallel with decrease in cytotoxic activity of it. On the other hand, the cytotoxic activity of a partially purified preparation, freed from adenosine deaminase-inhibitory activity, was not affected by the same treatment. Therefore, we expected that the culture extract contained both acid labile adenosine deaminase inhibitor and acid stable cytotoxic principle, and that the former contributed to potentiating cytotoxicity of the later.

Fifty liters of the culture extract were adjusted to pH 5.0 and applied on a charcoal column (8×40 cm). After washing the column with water, adenosine deaminase inhibitor fraction was eluted with 15% acetone prior to cytotoxic fraction. The inhibitor fraction was then applied on a column (4×60 cm) of high porous polymer HP-10 and eluted with water. The inhibitor fraction was furthermore applied on a DEAE-Sephadex A25 (Cl-type) column (3.5×65 cm), eluted with water, concentrated and lyophilized to give 170 mg of white powder. The UV-spectra of this inhibitor preparation are shown in Fig. 3. During incubation in 0.1 N HCl at room temperature, the spectrum of the isolated inhibitor changed gradually, in parallel with the decrease in inhibitory activity. These UV-spectral character-

FIG. 3. UV-spectral Change of the Isolated Adenosine Deaminase Inhibitor during Incubation in 0.1 N HCl at Room Temperature.

A, 0 min of incubation period; B, 10 min; C, 30 min; D, 60 min; E, 120 min; F, 240 min; and G, 310 min.

istics were quite identical with those of coformycin⁹⁾ or 2'-dCF,⁴⁾ both of which are potential adenosine deaminase inhibitors isolated from the family of *Actinomycetes*.^{4,9)} For identification of sugar moiety of the isolated inhibitor, 3'-deoxyadenosine, 2'-deoxyadenosine and isolated inhibitor were hydrolyzed in 0.1 N HCl at 100°C for 60 min. After removing hydrochloric acid, hydrolyzates were subjected to paper chromatography by using butanol-acetic acid-water (5:1:1). The *R_f* values and cysteine sulfuric acid reactions were 0.50 (+), 0.47 (+++), and 0.47(+++) for 3'-deoxyadenosine, 2'-deoxyadenosine and isolated inhibitor, respectively, suggesting that the isolated inhibitor was 2'-dCF. Assignments of NMR spectrum also supported this prediction. Therefore we concluded that the isolated adenosine deaminase inhibitor was 2'-dCF.

The cytotoxic fraction was eluted from the first charcoal column with 50% acetone. This fraction was concentrated *in vacuo* and allowed to stand overnight at 5°C. Needles formed were collected and recrystallized from hot water. Physicochemical characteristics of this preparation were as follows: UV λ_{max} nm: 258 (in 0.1 N HCl), 260 (in 0.1 M phosphate buffer, pH 7.0), and 260 (in 0.1 N NaOH). Anal. Found: C, 47.71; H, 5.13; N, 27.93. Calcd. for $C_{10}H_{13}N_5O_3$: C, 47.80; H, 5.22; N, 27.88%. Color reactions on paper chromatography: $NaIO_4$ -benzidine, negative; cysteine-sulfuric acid, positive. UV-spectra and color reactions of this preparation were in good agreement with those of deoxyadenosine. The signals of NMR spectrum, especially doublet at 5.82 ppm (1H, $J=2.5$ Hz) and multiplet at 2.05 ppm (2H) were assigned to be protons on C1' and C3' of the 3'-deoxyribose moiety, respec-

TABLE I. POTENTIATION OF CYTOTOXICITY OF CORDYCEPIN BY 2'-dCF

Cell	2'-dCF ($\mu\text{g/ml}$)	ID ₅₀ of Cordycepin ($\mu\text{g/ml}$)
L5178Y	0	10
	0.0001	3.2
	0.001	1.0
	0.01	0.032
	0.1	0.032
	1.0	0.032
L1210	0	10
	0.01	0.032
	0.1	0.032
	1.0	0.032
KB	0	32
	0.01	0.32
	0.1	0.32
	1.0	0.32

tively. Furthermore, IR spectrum of this preparation was quite identical with that of authentic 3'-deoxyadenosine (cordycepin). Therefore, this cytotoxic compound was concluded to be cordycepin.

This is the first report that fungi produced a potent adenosine deaminase inhibitor, 2'-dCF. In addition, this is the third case of simultaneous production of both an adenosine related antibiotic and a potent adenosine deaminase inhibitor. The first case was production of formycin with coformycin by *Nocardia interforma* and *S. kaniharaensis*³⁾ and the second was ara-A with 2'-dCF by *S. antibioticus*.⁴⁾ Probably there are some close relationships between the production of adenosine related antibiotics and adenosine deaminase inhibitors.

Activity of 2'-dCF to potentiate the cytotoxicity of cordycepin was examined. Fifty percent growth inhibition doses (ID₅₀'s) of cordycepin were 10, 10 and 32 $\mu\text{g/ml}$ against mouse leukemia L5178Y, L1210 and human KB cells, respectively. On the other hand, any remarkable cytotoxicity was not expressed by

2'-dCF alone up to 100 $\mu\text{g/ml}$. As shown in Table I, when more than 0.01 $\mu\text{g/ml}$ of 2'-dCF was present in medium, the apparent ID₅₀'s of cordycepin were as low as 0.032 and 0.32 $\mu\text{g/ml}$ against both types of mouse leukemia cells and human KB cells, respectively. The potentiating effect was found even at a concentration of 0.0001 $\mu\text{g/ml}$ of 2'-dCF. This potentiating effect of 2'-dCF might be primarily due to the protection against enzymic deamination of cordycepin.

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