ANTITUMOR ANTIBIOTICS PRODUCED BY *PENICILLIUM STIPITATUM* THOM

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(Received for publication August 25, 1973)

A new crystalline antibiotic designated PSX-1 has been isolated from the fermentation broth of *Penicillium stipitatum* THOM. Antibiotic PSX-1 is a neutral reddish substance melting at 33~34°C, containing only C, H and O. (λ_{max}. 207 and 270 nm in methanol). The other crystalline antibiotic identified as duclauxin was isolated from the filtrate of the above-mentioned strain. Both antibiotics showed an inhibitory effect on EHRLICH ascites carcinoma (EAC), lymphadenoma L-5178 and sarcoma 37.

The production of potential cancerostatic substances in a larger group of *Fungi imperfecti* was studied, using a method based on inhibition of incorporation of ^{14}C-labelled adenine and *l*-valine into EAC cells[1]. *Penicillium stipitatum* THOM was one of the tested cultures whose filtrates showed an observable activity on EAC cells. The known metabolites of this mould-tropolones-stipitatic acid[2], stipitatonic acid[3] and stipitalid[4] were isolated from the filtrates of given strain as well as the ethyl ester of stipitatic acid[5,6] showed no activity in the used screening test. The cancerostatic activity of filtrates detected, depended on the presence of some metabolites, yet unknown, of non-tropolone character.

Production and Isolation

A medium containing 9% sucrose, 1% corn-steep liquor (65% dry weight), 0.2% $NaNO_3$, 0.1% KH_2PO_4, 0.05% KCl, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.001% $FeSO_4 \cdot 7H_2O$ adjusted to pH 6.3 was found to be suitable for the growth of inoculum. Strain CBS 375.48 was cultivated in 100 ml of the medium, inoculated with 2.5 ml spore suspension, and placed in 500 ml shake flasks at 28°C for 42 hours. Four hundred ml of vegetative inoculum thus prepared was inoculated in 10 liters of the same medium in a small fermentor of 20-liter volume and cultivated for 30 hours at 28°C with aeration of 7.5 liters/min. and stirring at 300 r. p. m. Sixteen liters of the culture thus obtained were inoculated into 300 liters of medium containing 5% glucose, 0.2% $NaNO_3$, 0.1% KH_2PO_4, 0.5% KCl, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.001% $FeSO_4 \cdot 7H_2O$ adjusted pH to 6.3 in a stainless steel fermentor of 500-liter volume. The fermentation was continued for 120 hours at 28°C with aeration of 225 liters/min. and stirring at 220 r. p. m.

The mycelial cake was collected by filtration. The clear filtrate (190 liters, pH 4.0) was stirred for 30 minutes with 90 liters of tetrachloromethane at 22~24°C. The tetrachloromethane layer was separated, clarified by centrifugation and dried by filtration through anhydrous Na_2SO_4. The obtained solution was concentrated under reduced pressure to 500 ml and the concentrate was allowed to stand at 5°C overnight. From the concentrated extract the substance X-2 with a strong

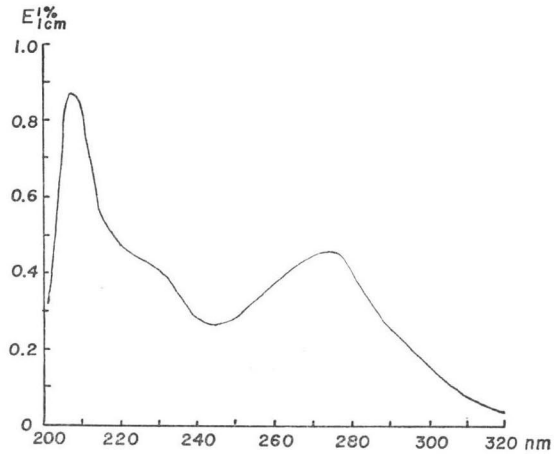
cytotoxic activity crystallized as a pale yellow powder (5.0 g). The crude substance was purified on a column with silica gel and 1.8 g pure colorless crystalline antibiotic was obtained and identified as duclauxin[7].

The culture filtrate remaining after tetrachloromethane extraction was stirred three times with 80 liters of chloroform in the same procedure as given above. The extracts were combined and the solvent removed under reduced pressure. One hundred and twenty ml of red-brown syrup (33 % dry weight), was purified by column chromatography on silica gel: Forty ml residue were put on the top of the column (ϕ 35 mm) with silica gel (300 g silica gel "L" Lachema), which was gradually eluted with a mixture of chloroform-methanol 100 : 5 (rate of elution 2 ml/min.) and the fractions of 20 ml were collected. The TLC on silica gel plates (Silufol^{R}) in the systems chloroform-methanol 100:5 and 10:1, with subsequent detection by $FeCl_3$ and $KMnO_4$ solution were used for estimating the presence of active substance. The biological activity of single fractions was simultaneously evaluated from the point of view of inhibition of incorporation of the ^{14}C-labelled adenine and *l*-valine into EAC cells[1]. The fractions Nos. 35~40 effective only on EAC cells containing mainly a spot with R_f 0.5 in chloroform-methanol 10 : 1 system were combined and evaporated *in vecuo*. In this procedure 8.3 g of active fraction was obtained in the form of reddish oil, which did not crystallize standing some days at 0°C. The viscous residue (oil) was again purified by the same procedure. The collected fractions containing only the spot R_f 0.5 were combined evaporated under reduced pressure as described above. A final yield of 2.8 g substance in the form of reddish syrup was obtained from which the active antibiotic in the form of faintly reddish plates was crystallized.

Physical and Chemical Properties

Antibiotic PSX-1 was obtained as faintly reddish crystals, melting at 33~34°C. The optical rotation is $[\alpha]_D^{22}+182°$ (*c* 1.0, chloroform). The molecule contains carbon, hydrogen and oxygen. The ultraviolet absorption spectrum of PSX-1 substance is shown in Fig. 1, indicating two maxima of 207 and 270 nm (with $E_{1cm}^{1\%}$ 88 and 45 in methanol). The infrared absorption spectrum in a potassium bromide pellet is given in Fig. 2. The antibiotic is readily soluble in chloroform, moderately in acetone, methanol and ethanol, slightly in benzene. It is insoluble in water and petroleum ether. When the antibiotic was examined by thin-layer chromatography using silica gel plates (Silufol^{R}) in the solvent system chloroform-methanol 10 : 1 a single spot detected with 2.4 DNPH and $KMnO_4$ was observed. When the TLC are allowed to stand for 30~60 minutes in the open air the antibiotic can be detected as a deep red spot. The substance gives no clolor reaction with $FeCl_3$ and diazoted benzidine. Substance PSX-1 is moderately stable, it decomposes partially in the open air (red spot). In the solution methanol, ethanol and acetone after standing or after

Fig. 1. Ultraviolet absorption spectrum of PSX-1 (in methanol)



heating some reddish spots R_f 0.61 and 0.74 occur TLC in silica gel in system chlorofom-methanol 10 : 1.

Biological Properties of PSX-1 and Duclauxin

The potential antitumor effect of PSX-1 and duclauxin was evaluated *in vitro* using EHRLICH ascites carcinoma, lymphadenoma L-5178 and sarcoma 37. The effect was evaluated according to the decrease of the amount of nucleic acids in the tumor cells[8] (Table 1). Both antibiotics suppressed the growth of HeLa cells and the cytotoxic effect was estimated by a modified OYAMA and EAGLE method[9]. The growth of mouse embryonic fibroblasts (LWF) as well as growth of malignant transformed cells by ROUS sarcoma virus were inhibited, when the substance PSX-1 was added to the growth medium.

Fig. 2. Infrared absorption spectrum of PSX-1 (in KBr tablet)

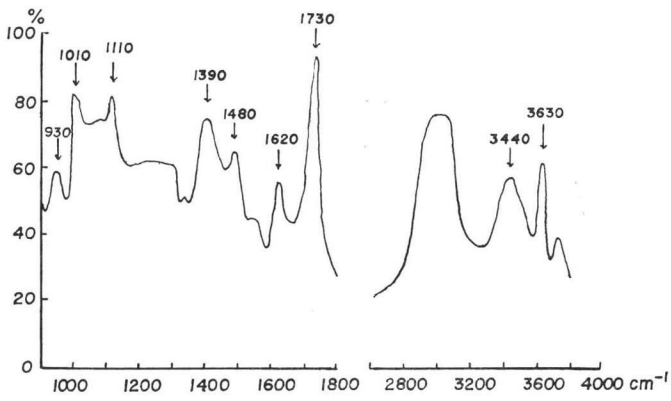


Table 1. Antitumor activity of antibiotic PSX-1 and duclauxin *in vitro*

Type of tumor	ED_{50} (μg/ml)	
	PSX-1	duclauxin
EHRLICH ascites carcinoma*	0.58	20.0
Lymphadenoma L-5178*	0.78	20.0
Sarcoma 37*	1.50	=
ROUS sarcoma	50.00	=
HeLa	2.0	50.0

* The results were obtained by Dr. L. P. IVANITSKAYA, Institute of New Antibiotics, Academy of Medical Sciences, Moscow, USSR.

Both substances caused the inhibition of incorporation of ^{14}C-labelled precursors of proteins and nucleic acids into EAC cells[1] (Fig. 3). The RNA synthesis in EAC cells was more inhibited than DNA synthesis when the substance PSX-1 was present in suspension cells (Fig. 4).

Antibiotic PSX-1 and duclauxin were slightly active against *Bacillus subtilis* (in concentration 250 μg/ml) and inactive against *Escherichia coli*, *Euglena gracilis* and *Astasia longa*. PSX-1

Fig. 3. Inhibition of incorporation of ^{14}C-labelled precursors into EAC cells by PSX-1

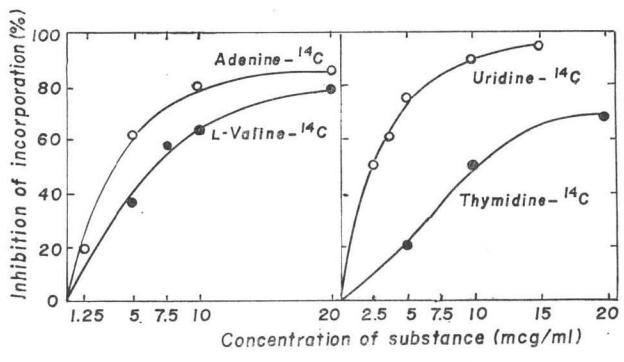
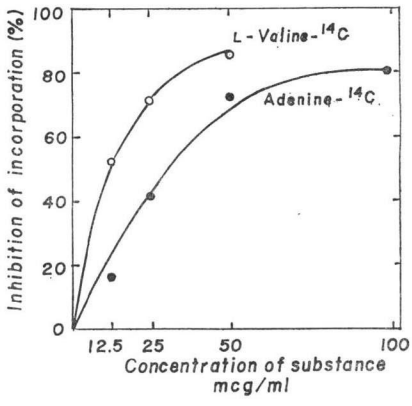


Fig. 4. Inhibition of incorporation of ^{14}C-labelled precursors into EAC cells by duclauxin (X-2)



inhibited the growth of *Candida pseudotropicalis* in concentration 50 μg/ml and the growth of *Trypanosoma cruzi* in concentration 100 μg/ml.

Discussion

Strain *Penicillium stipitatum* CBS 375.48 produced about 15 metabolites into the filtrate. More than ten substances with non-tropolone character can be found in the chloroform extracts, using TLC chromatography. These substances differ from the metabolites which have the structure of tropolones and which in the solvent systems used, remain at the beginning of the chromatogram, and most of them can be detected with $FeCl_3$ and diazoted benzidine. The isolated antibiotics PSX-1 and duclauxin are the main components with antitumor activity, though antitumor effect was shown also by some other fractions. The metabolites found in these fractions are present in the medium in small concentrations and their isolation is rather difficult and they have not yet been isolated.

Duclauxin and PSX-1 were found in the filtrates of the culture growing in various media. CZAPEK-DOX medium with glucose proved, to be the most suitable medium for their preparation. However, in this medium the strain produces only as mall amount of stipitatonic acid and stipitalid, which are extracted together with active substances into chloroform.

The physico-chemical properties of PSX-1 showed that the substance cannot be identical with the known tropolone metabolites[15] or with duclauxin, a modified phenalenone[10,11] produced by *Penicillium stipitatum*. The antibiotic differs from all non-tropolone metabolites produced by *Penicillium stipitatum*[12,13,14,15,16]. According to the physico-chemical properties the substance is probably not identical with any of the known and described fungel metabolites[17,18].

The authors are grateful to Mrs. M. ADAMKOVA and J. STROSSOVA for technical assistance through this work.

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