ASCOCHLORIN, A NEW ANTIBIOTIC, FOUND BY PAPER-DISC AGAR-DIFFUSION METHOD. I

ISOLATION, BIOLOGICAL AND CHEMICAL PROPERTIES OF ASCOCHLORIN

(STUDIES ON ANTIVIRAL AND ANTITUMOR ANTIBIOTICS. I)

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(Received for publication May 8, 1968)

A new antibiotic, ascochlorin, $C_{23}H_{29}O_4Cl$, was obtained from the filter cake of the fermented broth of *Ascochyta viciae* LIBERT. Ascochlorin inhibits plaque formation of both DNA and RNA viruses in the agar-diffusion plaque-inhibition method. However, it showed no antiviral activity by the tube culture method. It showed cytotoxicity to chick embryo fibroblast monolayer and HeLa cells at a concentration of 0.3 mcg/ml in tube culture. The antibiotic was obtained in two forms, the physical and chemical properties of which are described.

Many antiviral agents[1] have been found by application of tissue culture methods to the screening of synthetic compounds. N, N-Anhydrobis-(β-hydroxyethyl)biguanide[2] thus selected is now used as a prophylactic agent against influenza. However, only a few antiviral antibiotics have been obtained. The purpose of this investigation is to obtain new antiviral and antitumor antibiotics by utilizing tissue culture methods in screening, to clarify mechanisms of action and to elucidate molecular structures.

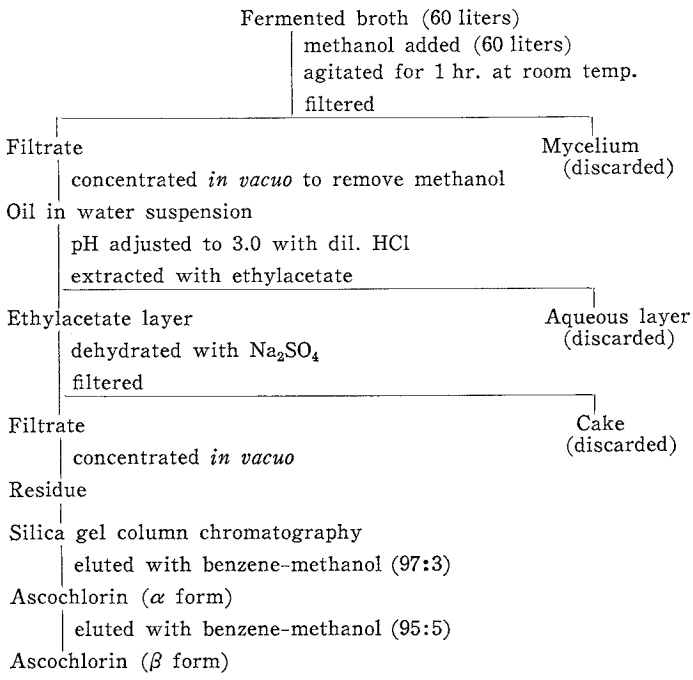
Using the paper-disc agar-diffusion method of HERRMANN[3] for screening, we obtained an antiviral antibiotic from the filter cake of the fermented broth of *Ascochyta viciae* LIBERT. The antibiotic is effective against both DNA and RNA viruses in the agar-diffusion plaque-inhibition method. Gliotoxin[4], tenuazonic acid[5], verrucarin A and brefeldin A[6] were reported to have antiviral activity among fungal metabolites, but the antibiotic obtained is clearly different from these and other known fungal metabolites in its biological and chemical properties.

Production and Isolation of Ascochlorin

It was found that *Ascochyta viciae* LIBERT produced an antibiotic effective against Newcastle disease virus strain Miyadera (NDV) growing on chick embryo fibroblast monolayer (CEF). The active material was found in the mycelium and was extracted with acetone or chloroform. *Asc. viciae* is a type culture of our laboratory that

belongs to Fungi Imperfecti and the genera *Phyllosticta*, *Phoma* and *Septoria* are in the same family. The fungus is a plant pathogen which causes black spots on the host plants. The metabolic products of the genus *Ascochyta* have been investigated and ascotoxin, a toxin of *Asc. imperfecta* PECK[7], is known as a specific metabolite for the fungus.

Fig. 1. Isolation of ascochlorin



The acetone extract of the mycelium showed inhibitory activity against *Candida albicans* in paper-disc agar-diffusion assay. The inhibition zones were turbid due to inhibition of the surface growth without inhibition of the inner growth. The activity against *C. albicans* reflected antiviral activity and the two biological activities showed in a spot the same Rf values in bioautograms when the extract was tested by thin-layer chromatography with various solvent systems. Therefore, an agar-diffusion assay using *C. albicans* was utilized for the fractionation and isolation of the active principle.

The isolation process is demonstrated in Fig. 1. Ascochlorin was obtained in two tautomeric forms designated α and β. The fungus was aerobically cultured in a fermentor at 26.5°C for 4 days. The medium consisted of glucose, peptone and yeast extract as chief nutrients. Although in a preliminary experiment the active principle was found to be present only in the mycelium, it was difficult to collect the mycelium by filtration because the fungus grew in minute pellets. Three volumes of methanol were added to the fermented broth to extract the active principle and to coagulate the mycelium. After the filtration the filtrate was concentrated *in vacuo* to remove methanol and the aqueous concentrate was adjusted to pH 3 by adding dil. HCl and extracted with ethylacetate. The ethylacetate layer, after dehydration with anhydrous sodium sulfate, was concentrated *in vacuo* to remove the solvent. Fractionation with silica gel chromatography yielded an oily residue. The active principle was eluted with benzene-methanol (97 : 3) and crystallized as colorless needles from the combined active fractions by evaporating the solvent *in vacuo*. The antibiotic is readily purified by recrystallization from methanol. We named ascochlorin because it was a metabolite of Ascochyta with a positive BEILSTEIN reaction which showed the presence of chlorine[8]. Later, it was found that its tautomer was present in the mycelial extract and was obtained as a colorless oil by elution of the column with a more polar solvent

benzene-methanol (95 : 5). Ascochlorin eluted with benzene-methanol (97 : 3) was designated α and the tautomer β.

Biological Properties of Ascochlorin

1. Antiviral Activity

Table 1 demonstrates the dose-response relationship of ascochlorin (α form) in the plaque inhibition method using CEF as the host cells. Plaque formation by herpes simplex virus strain HF (HSV) was inhibited as well as NDV so that both DNA and RNA viruses are involved in the antiviral spectrum. Although the plaque inhibition zone was not so large even at the highest concentration in this experiment (3,200 mcg/ml), a plaque inhibition zone of 25 mm in diameter was still shown when the original solution was diluted to 6 mcg/ml. In this system the cytotoxic zone appears inside of the plaque inhibition zone when the concentrations of a test material are high. Cytotoxicity of ascochlorin to CEF was low but was observed at concentrations higher than 200 mcg/ml. The index, that is, the ratio of the minimum effective concentration to the minimum cytotoxic concentration was >16. Only a few antiviral compounds show the indexes above 10 in this system. Ascochlorin was ineffective against HSV and vaccinia virus strain DIE in another antiviral activity assay system, the tube culture method. This method measures the suppression of the cytopathic effect (CPE) of the virus on the host cells in the presence of a test material. The susceptibility of HeLa cells to the toxic effect of ascochlorin was tested in triplicate tubes. After 24 and 48 hours' exposure to ascochlorin, the cell layers were vitally stained with neutral red, graded for degeneration and LD_{50} was calculated. LD_{50} of the antibiotic for HeLa cells was approximately 0.3 mcg/ml so that the experiments were carried out to determine an effective concentration in 2-fold serial dilutions starting from 0.3 mcg/ml. Ascochlorin did not suppress the cytopathic effect of vaccinia virus strain DIE and HSV at any dose, and higher doses of ascochlorin caused a drop in pH in HeLa cell cultures. The drop apparently occurs because the antibiotic stimulates the cells to maintain a high rate of glycolysis and to produce an excess of lactic acid in comparison to the control. In this respect ascochlorin might belong to the same class of antibiotics as ossamycin[9] and peliomycin[10].

Table 1. Antiviral activity of ascochlorin (α form) in agar-diffusion plaque-inhibition method

Concentration (mcg/ml)	Antiviral activity			
	NDV		HSV	
	CTZ	PIZ	CTZ	PIZ
	mm	mm	mm	mm
3,200	26	35	28	37
800	27	35	28	36
200	18	35	12	30
50	—	30	—	30
12	—	28	—	28
6	—	25	—	25

Antiviral activity was expressed as diameter of plaque-free protected zone (plaque-inhibited zone; PIZ) together with inner cytotoxic zone (CTZ). The viruses used were Newcastle disease virus strain Miyadera (NDV) and herpes simplex virus strain HF (HSV).

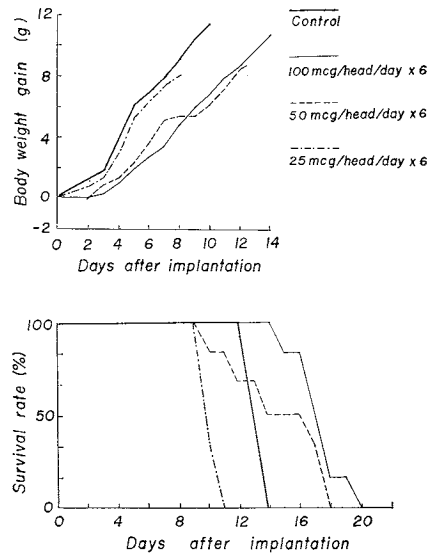
2. Antitumor Activity

The antitumor activity of ascochlorin *in vivo* was determined using EHRLICH ascites tumor-mouse system. Preliminary tests to determine the acute toxicity of ascochlorin were carried out by intraperitoneal injection. The LD_{50} for mice was found to be 20 mg/kg.

Treatment was initiated 24 hours after implantation of 2×10^6 EHRLICH ascites tumor cells, ascochlorin being given intraperitoneally once daily in a total volume 0.2 ml. Control mice that had been implanted with the tumor received an equal number of injections of the diluent, phosphate buffer saline.

The results are demonstrated in Fig. 2. A moderate antitumor activity was observed at a dose of 100 mcg per mouse for 5 days judging from the survival and body weight gain curves. In this experiment ascochlorin was found to be inflammatory to skin as are actinomycins and chromomycins.

Fig. 2. Antitumor activity of ascochlorin (α form)



Chemical Properties of Ascochlorin

Ascochlorin is readily soluble in most organic solvents such as acetone, chloroform, ethylacetate, ether and benzene; soluble in methanol and ethanol; slightly soluble in hexane and water. The crystalline form has no definite melting point: its crystals began to turn yellow at 120°C and melted at 153~154°C with decomposition. The molecular formula, $C_{23}H_{29}O_4Cl$, was assigned for ascochlorin on the basis of microanalyses and the molecular weight, 404, determined by mass spectrometry. Presence of chlorine was suggested by positive BEILSTEIN reaction and confirmed by elementary analysis and a characteristic P+2 peak (m/e 406) in the mass spectrum (Fig. 3).

Ultraviolet absorption maxima of the α-form of ascochlorin were λ_{max} 240 (39,000),

Fig. 3. Mass spectrum of ascochlorin (α form)

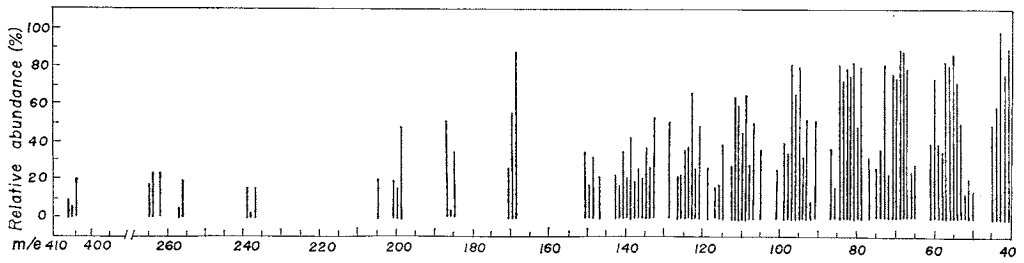


Fig. 4. IR spectrum of ascochlorin (α form)

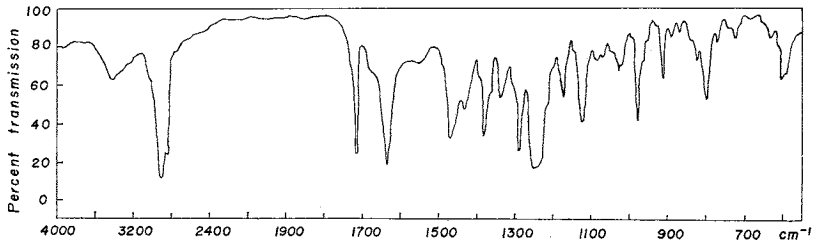
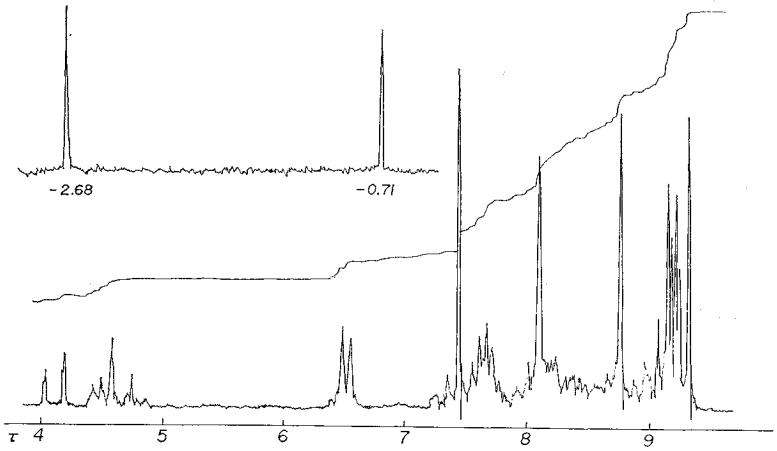


Fig. 5. NMR spectrum of ascochlorin (α form)



λ_{max} 290 (11,000) and λ_{max} 346 (10,900) in methanol.

Tautomerism of α and β forms was confirmed by thin-layer chromatography (TLC) using purified α form. After thin-layer chromatography silica gel in benzene-methanol (4 : 1), two spots with the same degree of antiviral activity appeared at Rf 0.92 and 0.67. The two spots were separately removed and after extraction of the silica gel with acetone, the extracts were again chromatographed with the same solvent system. The two spots were still present in each case, indicating a tautomeric interconversion of the ascochlorin forms. Also, the active principle could be divided into two fractions when the acetone extract of the mycelium was fractionated by silica gel column chromatography. The α form was the faster moving fraction which was eluted with benzene-methanol (97 : 3) and the β form eluted with benzene-methanol (95 : 5). The β form was obtained as an oil by evaporating the solvent and showed the same infrared absorption spectrum and molecular weight, 404, as the α form. The infrared absorption spectrum of the α form is shown in Fig. 4. The presence of hydroxyl, carbonyl and vinyl ether was observed in the IR. Acetylation of the α form with pyridine-acetic anhydride yielded a monoacetate that showed no band attributable to hydroxyl grouping, whereas the OH band still remained in the IR of the β form monoacetate.

The nuclear magnetic resonance spectrum is shown in Fig. 5. It shows two signals corresponding to single protons, respectively, at τ −0.68 and −2.68.

The structure study is under way and will be reported separately.

Experimental

Fermentation : *Ascochyta viciae* LIBERT was aerobically grown in a 100-liter tank containing 60 liters of the medium composed of (w/v, %) glucose 5, peptone 0.5, yeast extract 0.2, NH_4Cl 0.1, KH_2PO_4 0.06, $MgSO_4 \cdot 7H_2O$ 0.04 and $CaCO_3$ 1 (pH not adjusted). The tank was inoculated with 300 ml of *Asc. viciae* and stirred at 300 rpm and aerated 40 liters/min. for 4 days at 27°C.

Methanol (180 liters) was added to the fermented broth and stirred for 1 hour to extract ascochlorin from the mycelium. After filtering the coagulated mycelium with

diamatoceous earth, the filtrate was concentrated *in vacuo* to remove methanol. To the residual suspension (oil in water) was added sodium chloride (2.5 kg) and the suspension was adjusted to pH 3 with dil. HCl. The suspension was extracted three times with ethylacetate (10 liters) and the combined extracts were dried over anhydrous sodium sulfate. Sodium sulfate was filtered off and the filtrate was concentrated *in vacuo* to a small volume. A tarry brown oil (52 g) remained after evaporating the solvent *in vacuo*.

Isolation of the α form of ascochlorin: The tarry brown residue (52 g) was fractionated through the column (100 mm × 36 mm) of silica gel (120 g, Mallinckrodt 100 mesh) and Celite 545 (30 g) suspended in benzene. The column was developed with benzene (500 ml) and then ascochlorin was eluted with the mixed solvent, benzene–methanol (97 : 3) (1 liter). The fractions with inhibitory activity against *C. albicans* were collected and concentrated *in vacuo* to thick syrup. Ascochlorin (350 mg) crystallized as needles when the syrup was kept standing overnight at 5°C. Purified material (210 mg) was obtained by recrystallization from methanol (7.5 ml), mp 153~154°C (decomposition), molecular weight, 404 from mass spectroscopy.

Analysis. Calcd. for $C_{23}H_{29}O_2Cl$: C 68.35, H 7.18, Cl 8.62.
Found: C 68.59, H 7.36, Cl 8.42.

Isolation of the β form of ascochlorin: After elution of the α form the column was eluted with benzene–methanol (95 : 5) (1 liter). The β form formed larger growth-inhibitory zones against *C. albicans* than the α form in the agar diffusion assay. It was purified through column chromatography several times with the mixed solvents, benzene–methanol and chloroform–methanol, and was obtained as a colorless oil. Molecular weight, 404, from mass spectrum.

Antitumor activity: Mice strain *ddY* 5 weeks old weighing 18~22 g were used to determine acute toxicity and antitumor activity. Mice implanted with 2×10^6 EHRLICH ascites tumor cells were treated once daily for 5 consecutive days by intraperitoneal injection of ascochlorin suspended in 0.2 ml of phosphate buffer saline.

Antiviral activity: Paper-disc agar-diffusion method was used throughout the investigation. The viruses used were Newcastle disease virus strain Miyadera, herpes simplex virus strain HF and vaccinia virus strain DIE.

Acknowledgment

The authors are indebted to Mr. AIZAWA for the infrared, ultraviolet and NMR spectra, and to the members of the analytical laboratory of this department for microanalyses. They are also grateful to Mr. Y. SHIDA for mass spectroscopy.

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