# A NEW ANTIBIOTIC, 1-(*p*-HYDROXYPHENYL)-2,3-DIISOCYANO-4-(*p*-METHOXYPHENYL)-BUTA-1,3-DIENE. I

## ISOLATION AND BIOLOGICAL PROPERTIES

(STUDIES ON ANTIVIRAL AND ANTITUMOR ANTIBIOTICS. II)

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A new antibiotic, 1-(*p*-hydroxyphenyl)-2,3-diisocyano-4-(*p*-methoxyphenyl)-buta-1,3-diene, was isolated from the mycelium of *Dichotomomyces albus* SAITO in crystalline form by utilizing the paper disc-agar diffusion plaque-inhibition method. The antibiotic inhibits the plaque formation of Newcastle disease virus infected on primary chick embryo fibroblast cell monolayer. It is active against some gram-positive and gram-negative bacteria, including *Shigella flexneri* and *Proteus vulgaris*.

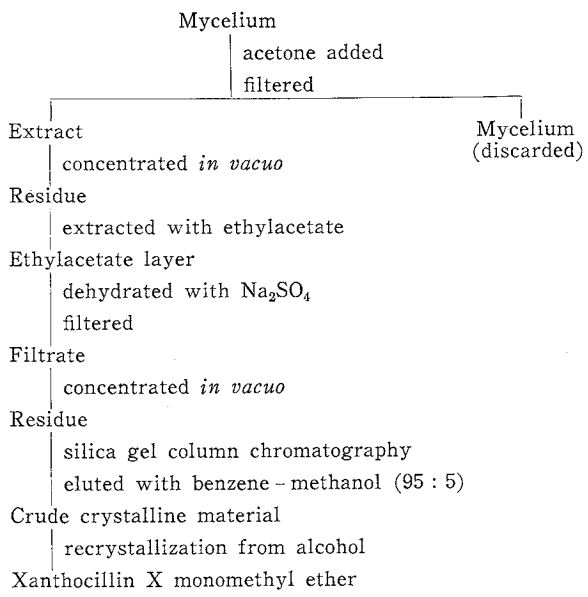
In our antiviral antibiotic screening using the paper disc-agar diffusion method of HERRMANN's[1], an acetone extract of the mycelium of *Dichotomomyces albus* SAITO showed antiviral activity against Newcastle disease virus strain Miyadera (NDV) together with some cytotoxicity. *Dich. albus*, belonging to Fungi Imperfecti, had been isolated by SAITO and preserved as a type culture in the Institute for Fermentation in Osaka. The active principle was isolated in crystalline form from the mycelium through silica gel column chromatography and the biological and chemical properties were investigated. Structural studies which identified the material as 1-(*p*-hydroxyphenyl)-2,3-diisocyano-4-(*p*-methoxyphenyl)-buta-1,3-diene (xanthocillin X monomethyl ether) will be reported separately.

### Production and Isolation

In primary screening using the paper disc-agar diffusion method[1], an acetone extract of the mycelium of *Dichotomomyces albus* SAITO showed antiviral activity against NDV. The extract inhibited the growth of *Bacillus subtilis* IAM 1026, *Escherichia coli* IAM 1264 and *Candida albicans* IAM 4888 in the agar diffusion assay. The screening medium contained glucose, peptone and yeast extract as the main nutrients. The growth rate of the fungus is somewhat slow, taking 7 days for maximal production of the antibiotic in shake culture. The fungus grows as a hard and voluminous mycelial mass, light yellow in color. The culture filtrate also showed strong inhibitory activity against *Bacillus subtilis* in an agar-diffusion assay due to the co-production of gliotoxin and gliotoxin acetate in the culture medium. However,

these antibiotics showed no plaque-inhibitory activity against NDV, although they were strongly cytotoxic to CEF[2]. The active principle was present only in the mycelium due to its insolubility in water. Inhibitory activity against *B. subtilis* reflected antiviral activity since both biological activities showed the same Rf values in bioautograms using silica-gel thin layer chromatography in various solvent systems. The agar-diffusion assay using *B. subtilis* as the test organism was utilized to speed up fractionation and isolation.

Fig. 1. Isolation and crystallization




The course of isolation and crystallization of the active principle is outlined in Fig. 1. A subculture was made at 27°C on malt agar slants, and transfers were carried out into test tubes of 20 mm diameter and 200 mm length containing 15 ml of medium containing (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 in tap water at pH 6.0~6.5. The tubes were incubated at 27°C for 5 days in a test tube shaker. A total of 15 ml of inoculum was added to a 5-liter Ehrlenmeyer flask equipped with baffles containing 0.8 liter of the medium with the same composition as the inoculation medium. Flasks were incubated at 27°C on rotary shaker for 7 days. The pH of the medium decreased the first 48 hours to 5.1, when calcium carbonate disappeared completely from the medium. After 7 days, the fermented broth was filtered and the filter cake containing the mycelium was collected. The cakes were extracted with acetone overnight at room temperature and the extract obtained by filtration was concentrated under reduced pressure to remove acetone. The residual suspension was extracted with ethylacetate and after dehydration of the ethylacetate layer with anhydrous sodium sulfate, the extract was concentrated under reduced pressure to dryness, yielding yellowish semi-solid with all of the antiviral activity of the original extract. Silica-gel column chromatography was applied to the residue. The active principle was eluted from the column by the mixed solvent, benzene - methanol (95 : 5), and crystallized as long needles with light yellow color when the tubes containing the active principle were kept standing at 5°C overnight. The antibiotic is readily recrystallized from alcohol or acetone as long needles. The yield is 30~40 mg/liter of the fermented broth. The antibiotic is soluble in acetone, ethylacetate and ether; slightly soluble in benzene, chloroform and alcohol; insoluble in hexane and water. The antibiotic showed no definite melting point: the crystals turned to dark yellow at 120°C and decomposed at 166°C.

## Biological Activities

Antiviral Activity:

Antiviral activity in the paper disc-agar diffusion system was determined with two-fold serial dilution experiments starting from 16.5 mg/ml. The antibiotic effectively inhibited plaque formation of NDV infected on CEF as shown in Table 1. Plaque-

Table 1. Antiviral activity of xanthocillin X monomethyl ether

| Concentration (mcg/ml) | Antiviral activity | |
|---|---|---|
| | CTZ (mm) | AVZ (mm) |
| 16,500 | — | 22 |
| 8,250 | — | 22 |
| 4,125 | — | 20 |
| 2,062 | — | 20 |
| 1,031 | — | 20 |
| 515 | — | 15 |
| 257 | — | 15 |
| 128 | — | 15 |
| 64 | — | 15 |
| 32 | — | — |

The virus used was Newcastle disease virus strain Miyadera and the cells were primary chick embryo fibroblast. Antiviral activity was expressed as the diameter of plaque-free protected zones (AVZ) and inner cytotoxic zone (CTZ).

Table 2. Antimicrobial spectrum of xanthocillin X monomethyl ether.

| Organisms | Assay system | Minimum inhibitory concentration (mcg/ml) |
|---|---|---|
| *Staphylococcus aureus* | A | 1.2 |
| *Staph. aureus* | T | 0.3 |
| *Sarcina lutea* | A | 5.1 |
| *Micrococcus pyogenes* | A | 2.6 |
| *Bacillus subtilis* | A | 2.6 |
| *Shigella flexneri* | T | 0.6 |
| *Pseudomonas aeruginosa* | A | >80 |
| *Proteus vulgaris* | A | 5.1 |
| *Xanthomonas oryzae* | A | >80 |
| *Escherichia coli* | A | 10.4 |
| *Candida albicans* | A | 5.1 |
| *Rhizopus oryzae* | A | 10.4 |
| *Saccharomyces cerevisiae* | A | >80 |
| *Hansenula anomala* | A | >80 |
| *Aspergillus niger* | A | >80 |
| *Piricularia oryzae* | A | 10.4 |

A: Agar streak method. T: Tube culture method.

inhibited zones were not large at the highest concentration because of the slow diffusion rate compared with brefeldin A and verrucarin A[3], but there was no cytotoxicity to CEF at any dose tested. Cytotoxity, however, was observed at concentrations of 3~6 mcg/ml in tube cultures. It is not clear why the antibiotic was non-toxic in the agar diffusion method, although it was relatively toxic in tube culture.

Susceptibility of HeLa cells to the toxic effect of the antibiotic was determined in triplicate tubes. After 24 and 48 hours exposure to the antibiotic, the cell layers were vitally stained with neutral red, graded for degeneration and $LD_{50}$ was calculated. $LD_{50}$ of the antibiotic was approximately 0.3 mcg/ml.

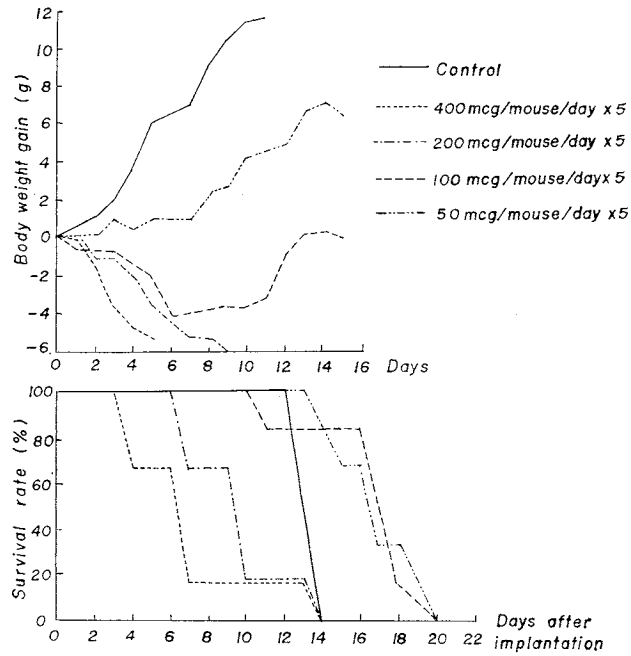
Determination of the inhibitory activity was carried out against vaccinia virus strain DIE and herpes simplex virus strain HF in two-fold serial dilution method starting from 0.3 mcg/ml. HeLA cell monolayers were used as host. The antibiotic was not so effective against these viruses in tube cultures because suppression of cytopathic effect (CPE) was observed only at a dose, 0.15 mcg/ml, slightly toxic to the host cells. Inhibition of CPE by the antibiotic always accompanied cytotoxicity to the cells and no antiviral activity was observed at any dose lower than 0.15 mcg/ml.

Antimicrobial Activity:

The antibiotic gave small growth inhibition zones in an agar diffusion assay,

diameter of which was smaller than 15 mm at any dose with a paper disc of 8 mm in diameter. However, when the minimal inhibitory concentration was determined in tube culture or agar streak methods, it was found to be a strong antimicrobial agent. The antimicrobial spectrum is wide, including both gram-positive and gram-negative bacteria and some fungi as shown in Table 2. The antibiotic inhibits the growth of some pathogenic bacteria such as *Shigella flexneri* and *Staphylococcus aureus* at low concentrations. Usefulness, however, might be limited due to the highly delayed toxicity *in vivo*.

Fig. 2. Antitumor activity of xanthocillin X methyl ether.




Antitumor Activity:

EHRLICH ascites carcinoma-mouse strain *ddY* system was used for determination of the antitumor activity. In a preliminary test, intraperitoneal administration of the antibiotic showed highly delayed toxicity to mice: toxic symptoms were shown by rapid decrease in body weight 2~3 days after the injections. Acute $LD_{50}$ calculated from the experiment was 40 mg/kg for intraperitoneal injection.

The antitumor experiments were carried out by a two-fold serial dilution method starting with a dose half of the acute $LD_{50}$. Treatment was initiated 24 hours after intraperitoneal implantation of $10^6$ EHRLICH ascites tumor cells into mouse strain *ddY* of 5 weeks age weighing 18~22 g. The antibiotic, suspended in phosphate buffer saline (pH 7.0), was intraperitoneally administrated once daily for 5 consecutive days. The activity was determined from the body weight gain and survival curves. The results are shown in Fig. 2. At doses of 400 and 200 mcg/mouse/day, the antibiotic caused a rapid decrease in body weight and led to death within several days. At a doses of 100 mcg/mouse/day or below, body weight gains were suppressed during treatment but the effect was temporary since the tumor began to grow as soon as administration was stopped. Thus, the antibiotic is ineffective against EHRLICH ascites tumor because prolongation of the life span of the tumor-bearing mice was not observed even if it was administrated at the optimal dose, 100 mcg/mouse/day.

## Discussion

The antibiotic we obtained is a new xanthocillin derivative, as reported elsewhere[4]. Xanthocillin X (XX) is a unique antibiotic containing two isocyano groupings[5] which was

found by BAIERSDORF and AHRENS[6] from the filter cake of the fermented broth of *Penicillium notatum*. XX is a broad spectrum antibiotic and is active against gram-positive and gram-negative bacteria including *Corynebacterium diphtheriae*, *Salmonella typhosa*, *Shigella dysenteriae*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Proteus vulgaris* at doses of 0.5~2.0 mcg/ml. Although XX is effective against some pathogenic bacteria *in vivo*, it is highly toxic; the $LD_{100}$ for guinea pigs was reported to be 50 mg/kg intracardially. When the antimicrobial spectrum of XX is compared with that of the antibiotic we obtained, minimal inhibitory doses against *Ps. aeruginosa* are different: XX inhibits the growth of the organism at a concentration of 2 mcg/ml, whereas the new antibiotic was inactive at 80 mcg/ml. Except for *Ps. aeruginosa* the spectrum of the antibiotic is in good accordance with that of XX.

This paper is the first report that a xanthocillin derivative exerts antiviral activity *in vitro*. The antibiotic effectively inhibits the plaque formation of NDV on CEF without cytotoxicity, whereas it shows cytotoxicity to CEF in tube cultures. The reason for the difference in the cytotoxicity in the two-assay systems is not clear. The antibiotic is not as active against vaccinia and herpes simplex viruses in tube cultures of HeLa cells as in the agar diffusion plaque inhibition method. Although viruses and host cells are different from those of the agar diffusion method, ineffectiveness in the tube cultures indicates that the antibiotic would not be expected to exert a prophylactic activity *in vivo*.

It is interesting that the $LD_{50}$ for HeLa cells is one tenth that of CEF. If HeLa cells are regarded as malignant cells and CEF as normal, this difference would have significant meaning. Thus, antitumor experiments were carried out using the EHRLICH ascites tumor-bearing mice. However, an unexpected result was obtained from this experiment: that is, the antibiotic could inhibit the tumor growth during the treatment but the tumor grew immediately after cessation of administration and no prolongation of the life-span of the tumor-bearing mice was observed. The ineffectivenss may be due to a cytostatic rather than cytocidal effect, or the EHRLICH ascites tumor cells may be resistant to the action of the antibiotic.

It has been considered that antitumor agents are closely related to antiviral agents because many antitumor agents possess antiviral activity *in vitro* or *in vivo*. Conversely, it is clear from the present work that an antiviral agent does not always show antitumor activity.

Acknowledgement

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