ORYZACHLORIN, A NEW ANTIFUNGAL ANTIBIOTIC

(STUDIES ON ANTIVIRAL AND ANTITUMOR ANTIBIOTICS. XVIII)

AKIKO KATO, TETSUJI SAEKI, SEIKICHI SUZUKI*, KUNIO ANDO,
GAKUZO TAMURA and KEI ARIMA

Laboratory of Microbiology, Dapartment of Agricultural Chemistry,
The University of Tokyo, Tokyo, Japan
*Research Laboratories, Chugai Pharmaceutical Co. Ltd., Tokyo, Japan

(Received for publication May 12, 1969)

A new antibiotic, oryzachlorin, $C_{26}H_{31}O_8N_2S_2Cl$ (λ_{max} 298 mμ in ethanol) has been isolated from *Aspergillus oryzae*. It strongly inhibits the growth of many yeasts but has no effect on bacteria or filamentous fungi. It also has antiviral activity *in vitro*.

In our screening for antiviral antibiotics, using the agar diffusion method[1], *Aspergillus oryzae* (AHLB.) COHN IAM-2613 was studied for the production of antiviral substances. In the first screening assay, this organism produced activity against Newcastle disease virus *in vitro* and cytotoxicity against chick embryo fibroblast cells. It also showed strong inhibition of growth against *Candida albicans*. No antibiotics from *Aspergillus oryzae* has previously been reported to have antiviral and anti-*Candida* activities. In this paper the production, isolation, purification, physico-chemical and biological properties of this antiviral and antifungal antibiotic are described.

Production and Isolation of Oryzachlorin

Preliminary experiments showed that sucrose is the best carbon source for production of the antibiotic. *Aspergillus oryzae* IAM-2613 was cultured in shake-flasks at 26.5°C for 4 days in the following medium: sucrose 5.0, peptone 0.5, yeast extract 0.2, KH_2PO_4 0.06, NH_4Cl 0.1, $MgSO_4 \cdot 7H_2O$ 0.04 and $CaCO_3$ 1.0% (w/v). The mycelium obtained by filtration of the culture was extracted with acetone and the extract was freed of solvent under reduced pressure. The residue was extracted with ethylacetate. The filtrate of the culture was extracted with ethylacetate several times until the extract was inactive against the indicator organism, *Candida albicans*. The combined ethylacetate extracts were dried over anhydrous sodium sulfate and concentrated *in vacuo* to obtain an oily residue. This residue was dissolved in a small amount of benzene and applied to a silica gel column, which was then eluted with benzene to remove inactive material. Active fractions were obtained by eluting the column with benzene - methanol (95 : 5). The active fractions were combined and, after the solvent was removed, were rechromatographed on a silica gel column with benzene-acetone. The active principle was eluted using the solvent mixture in a

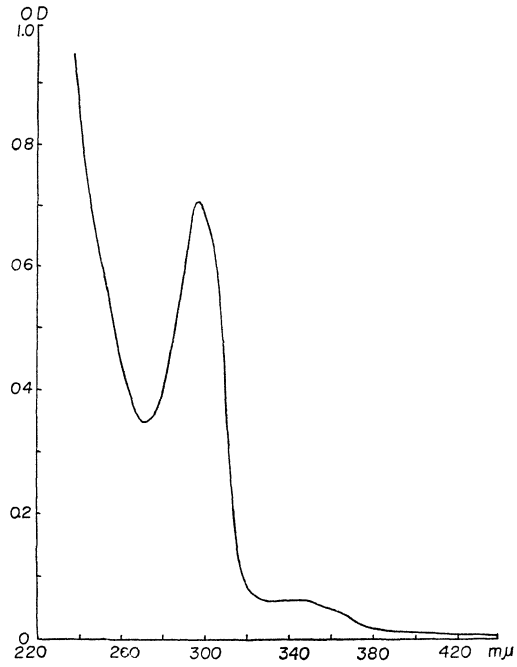
ratio of 9 : 1. Further purification was performed with chromatography on a Florisil (Floridin Co.) column. The active fractions were eluted with benzene - acetone (95 : 5) and concentrated under reduced pressure to obtain a yellow powder.

Physical and Chemical Properties of Oryzachlorin

Oryzachlorin obtained is soluble in methanol, acetone, chloroform and ethylacetate, sparingly soluble in benzene and petroleum ether and insoluble in hexane. Oryzachlorin is heat stable. Its activity against *Candida albicans* and Newcastle disease virus *in vitro* does not decrease after heating at 100°C for 30 minutes. It is also stable to ultraviolet irradiation for 30 minutes.

As shown in Fig. 1, oryzachlorin shows a maximum at 298 mμ ($E^{1\%}_{1cm}$ 117) in methanol. The infrared spectrum of the antibiotic in nujol mull is presented in Fig. 2, and suggests the presence of hydroxyl (3200 cm^{-1}), carbonyl (1720 cm^{-1}) and conjugated double bond (1622 and 1600 cm^{-1}). The nuclear magnetic resonance spectrum is shown in Fig. 3.

Fig. 1. Ultraviolet spectrum of oryzachlorin. (in methanol)



Anal. Found:
C 51.35, H 5.12, N 4.67,
S 10.62, Cl 6.80.

Calculated for $C_{26}H_{31}O_8N_2S_2Cl$:
C 52.17, H 5.18, N 4.68,
S 10.70, Cl 5.85.

Although the mass spectrum of oryzachlorin did not show its parent peak, a fragment peak at m/e 360 was accompanied by a relatively large peak at m/e 362 which indicates the presence of a chlorine atom in the molecule.

Oryzachlorin decolorizes potassium

Fig. 2. Infrared spectrum of oryzachlorin. (Nujol)

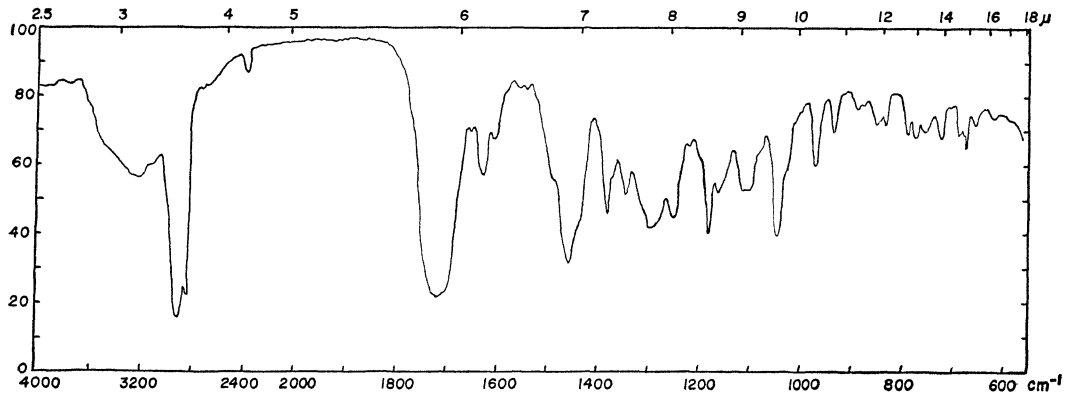
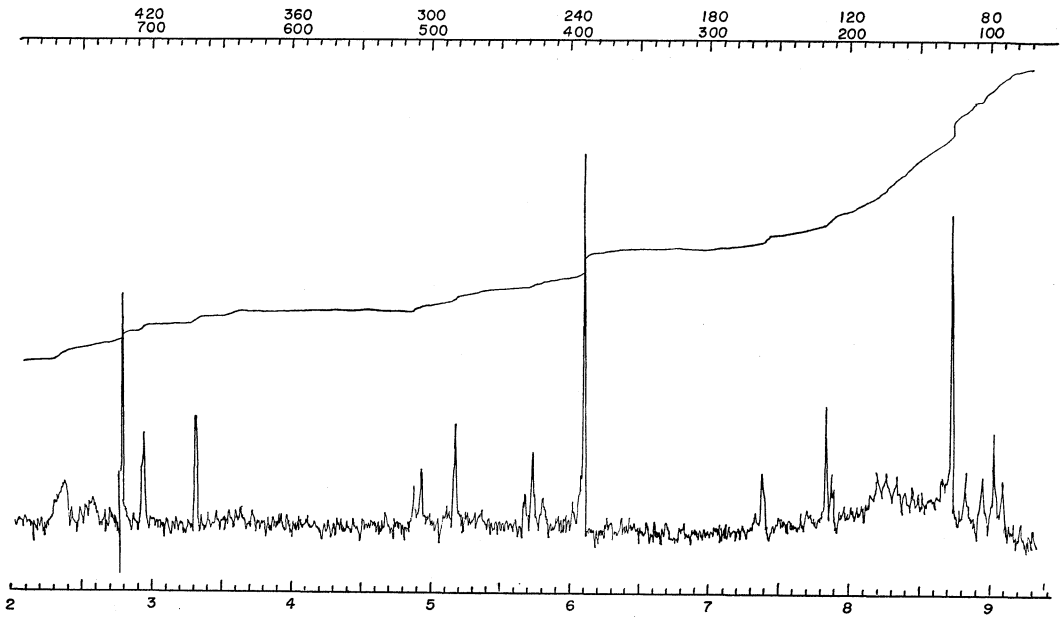


Fig. 3. NMR spectrum of oryzachlorin.



permanganate solution and gives a negative TOLLENS test.

Biological Activity of Oryzachlorin

The antimicrobial activity of oryzachlorin was studied using the agar dilution method. The minimal inhibitory concentrations observed are listed in Table 1. It shows specific inhibitory activity against *Cryptococcus* and no activity against bacteria and fungi. The antifungal activity against various strains of yeasts was further studied and the results are presented in Table 2.

The antiviral activity *in vitro* aaingt Newcastle disease virus was examined using the agar-diffusion method. Primary chick embryo fibroblast cell monolayers (CEF) were infected with the virus and overlaid with maintenance medium containing 1 % agar and neutral red. Antiviral activity was expressed as diameter of the plaque-free zone which appeared around the paper disks on CEF. Table 3 shows the dose-response relationships in this test. The antibiotic shows activity against Newcastle disease virus at concentrations of 15.6 mcg/ml. Its cytotoxicity on CEF appeared at 62.5 mcg/ml. The index in this assay system is thus 4. It also suppressed plaque formation of herpes and vaccinia viruses which are DNA viruses. The antitumor activity of

Table 1. Antimicrobial spectrum of oryzachlorin

Test organisms	M.I.C. (mcg/ml)
Staphylococcus aureus IAM-1058	>500
Bacillus subtilis IAM-1026	>500
Sarcina lutea IAM-1097	>500
Bacillus megaterium KM	100
Bacillus cereus IAM-1656	>500
Escherichia coli K-12	>500
Pseudomonas aeruginosa IAM-1202	>500
Proteus vulgaris IAM-1025	>500
Xanthomonas oryzae IAM-1657	>500
Candida albicans IAM-4888	0.3
Candida utilis IAM-4215	0.8
Aspergillus niger IAM-2093	>500
Rhizopus nigricans	>500

M.I.C.: Minimal inhibitory concentration by agar dilution method

oryzachlorin against EHRLICH ascites tumor in mice was studied. Mice, strain ddY, 5 weeks old and weighing 18~22 g were inoculated intraperitoneally with 2×10^6 tumor cells. Treatment was initiated 24 hours after inoculation using varied doses of oryzachlorin given by the same route. The treatments were continued once daily for 7 consecutive days. Life span and body weight gain of the treated mice were compared with those of the control mice. The results are given in Figs. 4 and 5.

Table 2. Antifungal activity of oryzachlorin

Test organisms	M.I.C. (mcg/ml)
Saccharomyces cerevisiae Hansen IAM-4512	>100
Saccharomyces rouxii Boutroux IAM-4028	12.5
Schizosacchromyces pombe IAM-4863	3.12
Pichia membranaefaciens IAM-4025	3.12
Hansenula anomala IAM-4213	3.12
Saccharomycodes ludwigii IAM-4380	1.6
Endomycopsis capsularis IAM-4307	0.4
Candida albicans IAM-4888	0.4
" IAM-4924	>100
" IAM-4905	6.25
Candida arborea IAM-4147	>100
Candida utilis IAM-4215	0.8
Candida japonica IAM-4185	3.12
Candida mycoderma IAM-4564	3.12
Candida pseudotropicalis IAM-4829	0.4
Candida tropicalis IAM-4862	>100
Candida krusei IAM-4801	3.12
Cryptococcus neoformans IAM-4788	3.12
Cryptococcus albidus IAM-4830	1.6
Trigonopsis colliculosa Hartmann IAM-4426	0.8
Rhodotorula glutinis IAM-4642	0.8
Rhodotorula rosa IAM-4929	3.12

Table 3. Antiviral activity of oryzachlorin by agar-diffusion method

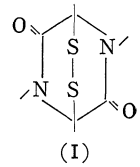
Concentration of oryzachlorin (mcg/ml)	Anti-NDV activity	
	Cytotoxic zone	Plaque inhibitory zone
500	23 mm	27 mm
250	17	20
125	16	19
62.5	12	15
31.25	—	13
15.6	—	12
8.0	—	—

Newcastle disease virus strain Miyadera (NDV) was used.

Discussion

Aspergillus oryzae (AHLB.) COHN IAM-2613 produces oryzachlorin, a new antibiotic which shows a specific inhibitory activity against yeasts, while it has no effect on most bacteria and filamentous fungi. Although tremendous number of antibiotics have been isolated and studied, very few of them are effective in the treatment of fungal infections. Only the polyene antibiotic show antifungal but not antibacterial activity. Oryzachlorin is as effective *in vitro* as the polyene antibiotics, nystatin and amphotericin B, against yeasts but physico-chemical studies of oryzachlorin indicates that it is not a polyene antibiotic. Moreover, oryzachlorin, unlike the polyenes, has no effect on filamentous fungi.

The empirical formula for oryzachlorin deduced from elemental analysis is $C_{26}H_{31}O_8\cdot N_2S_2Cl$. This formula, however, is tentative, because the analytical data do not completely agree with the theoretical data. A large fragment ion at m/e 360 in mass spectrum loses 64 mass units showing a peak at m/e 296. It is likely that it contains the epicithiapiperazinedione moiety (I) which is the common structure of gliotoxins[2] and acetylaranotin[3] (also named LL-S88)[4] recently reported as the antiviral agent produced by some fungi. The complete elucidation of the structure of oryzachlorin remains to be done.



(I)

Fig. 4. Antitumor activity of oryzachlorin.

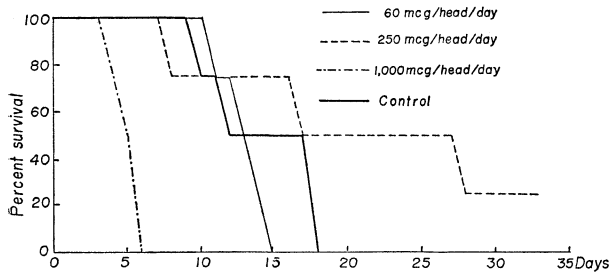
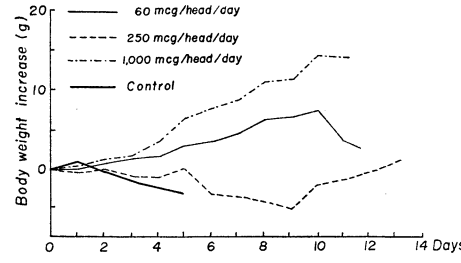


Fig. 5. Body weight change.



Acknowledgements

The authors wish to thank Roussel Uclaf for the tank fermentation of oryzachlorin. Thanks are also due to Mr. I. AIZAWA for measurements of IR and UV spectra, to Mr. Y. SHIDA for mass spectroscopy and to the members of the analytical laboratory in this department for microanalyses.

References

1) ANDO, K.; S. SUZUKI, G. TAMURA & K. ARIMA: Antiviral activity of mycophenolic acid. Studies on antiviral and antitumor antibiotics. IV. J. Antibiotics 21 : 649～652, 1968.
2) BELL, M. R.; J. R. JOHNSON, B. S. WILDI & R. B. WOODWARD: The structure of gliotoxin. J. Am. Chem. Soc. 80 : 1001, 1958.
3) NAGARAJAN, R.; L. L. HUCKSTEP, D. H. LIVELY, D. C. DELONG, M. M. MARSH & N. NEUSS: Aranotin and related metabolites from *Arachniotus aureus*. I. Determination of structure. J. Am. Chem. Soc. 90 : 2980～2982, 1968.
4) MILLER, P. A.; P. W. TROWN, W. FULMOR, G. O. MORTON & J. KARLINER: An epidithiapiperazinedione antiviral agent from *Aspergillus terreus*. Biochem. Biophys. Res. Commun. 33 : 219～221, 1968.