VERMICULINE, A NEW ANTIPROTOZOAL ANTIBIOTIC FROM *PENICILLIUM VERMICULATUM*

J. FUSKA, P. NEMEC and I. KUHR*

Department of Microbiology and Biochemistry, Faculty of Cheanoistry,
Slovak Polytechnical University, Bratislava, and
* Institute of Veterinary Medicine, Brno, Czechoslovakia

(Received for publication August 20, 1971)

A new crystalline antibiotic named vermiculine has been isolated from the fermentation broth of *Penicillium vermiculatum* DANGEARD. Vermiculine is a neutral colorless substance melting at 175~177°C with decomposition, containing only C, H and O. (λ_{max}. 222 mμ in methanol). The antibiotic inhibits growth of *Trypanosoma cruzi*, *Leishmania braziliensis* and some Gram-positive bacteria.

In the course of our screening program for antiprotozoal antibiotics produced by fungi[1,2], one of our strains, designated as 51-C_1, was chosen for its activity against *Trypanosoma cruzi* and *Leishmania braziliensis*. This strain, originally isolated from soil samples collected at Jáchymov, CSSR, was classified as *Penicillium vermiculatum* DANGEARD[3] and deposited in the Czechoslovak Collection of Microorganisms as No. CCM F 276.

From the culture broth of this strain a new antiprotozoal antibiotic, named vermiculine, was obtained in crystalline form. The present paper deals with the production, isolation and some of the physico-chemical and biological properties of vermiculine.

Production and Isolation

On examining culture conditions, a medium containing 9 % saccharose, 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 both the inoculum and production of vermiculine. Strain CCM F 276 was cultivated in 100 ml of the medium, placed in 500 ml shake flasks at 28°C for 42 hours. Two thousand ml of the culture thus obtained was inoculated in 250 liters of the same medium in a fermentor of 500-liter volume and cultivated for 24 hours at 27°C with aeration of 500 liters/min. and stirring at 220 r.p.m. Two hundred liters of the culture thus obtained were inoculated into 3,500 liters of the same medium in a stainless steel fermentor of 5,000-liter volume. The fermentation was continued for 120 hours at 28°C with aeration by 3,500 liters/min. and stirring at 180 r.p.m.

The mycelial cake was collected by filtration. The clear filtrate (1,800 liters, pH 3.5) was stirred for 30 minutes with 600 liters of chloroform at 24~26°C. The chloroform layer was separated, clarified by centrifugation and dried by filtration through anhydrous Na_2SO_4. The chloroform solution was concentrated under reduced

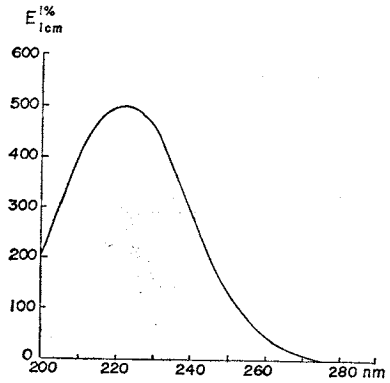
pressure to 500 ml and the concentrate was allowed to stand at 5°C, whereupon vermiculine crystallized as pale brown needles. A crude crystalline antibiotic (36.5 g) was obtained after leaving the solution overnight. The crude product was further purified by recrystallization from boiling acetic acid, having been simultaneously decolorised by activated carbon. A final yield of 27.5 g of pure vermiculine was obtained.

Physical and Chemical Properties

Table 1. The results of vermiculine on thin-layer chromatography using silica gel (Silufol^{R})

Solvent system	Rf
Chloroform - methanol (98 : 2)	0.71
Chloroform - acetone (8 : 2)	0.47
Benzene - acetone (7 : 3)	0.52
Benzene - acetone (8 : 2)	0.23
Benzene - methanol (9 : 1)	0.44
Benzene - acetic acid (1 : 1)	0.48
Ethylacetate - acetic acid (10 : 1)	0.59

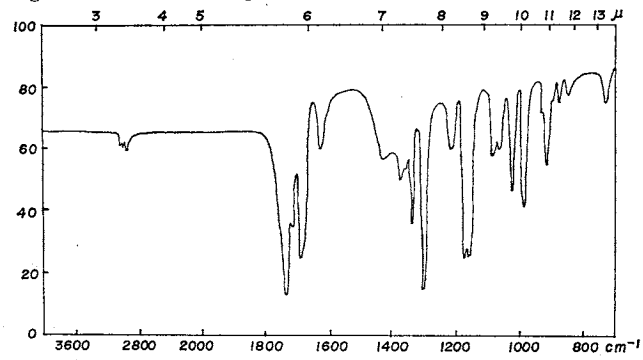
Fig. 1. Ultraviolet absorption spectrum of vermiculine (in methanol).



Vermiculine was obtained as colorless needle crystals with neutral properties. It melts at 175~177°C with decomposition. The optical rotation is $[\alpha]_D^{20}$ −12.5° (*c* 0.2, chloroform). When the antibiotic was examined by thin-layer chromatography using silica gel (Silufol^{R}) in many solvent system, a single spot detected with concentrated H_2SO_4 or $KMnO_4$ was observed (Table 1). This spot had the same Rf as we found using bioautography with *Bacillus subtilis* SDPC 1 : 220. Therefore, vermiculine has been shown to have only one component.

The molecule contains carbon, hydrogen and oxygen. Elemental analysis gives the following values: C 62.20, H 6.21, $O_{diff.}$ 31.59 %. The ultraviolet absorption spectrum of vermiculine is shown in Fig. 1, indicating a maximum of 222 mμ with $E_{1cm}^{1\%}$ 495 in methanol. The infrared absorption spectrum in a potassium bromide pellet is given in Fig. 2, showing maximum absorption wave numbers cm^{-1}: 3000, 2980, 2940, 1740, 1715, 1695, 1425, 1385, 1365, 1335, 1300, 1210, 1175, 1165, 1085, 1075, 985, 910, 875, 850 and 725. The antibiotic is moderately soluble in hot acetic acid (10 g/100 ml), chloroform (300 mg/100 ml at 25°C), slightly soluble in water and most organic solvents and insoluble in petroleum ether. It is readily soluble in cold HNO_3 and precipitates unchanged and pouring this solution into water.

Fig. 2. Infrared absorption of vermiculine (in KBr tablet).



Vermiculine with concentrated sulfuric acid gives a violet color. It dissolves slowly in alkaline solutions alcoholic or aqueous of KOH, NaOH, NH_4OH and pyridine, giving a brown-yellow coloration. Vermiculine gives positive TOLLENS, FEHLING, 2,4-DNPH and potassium permaganate reactions. It gives a positive ferric-hydroxamate test for ester and lactone groups and gives negative ferric chloride and Br_2 (in $CHCl_3$) tests. In chloroform solutions stored in the dark, vermiculine remains stable for several months. When exposed to daylight, it decomposes in solution and forms several substances as shown by thin-layer chromatography.

Biological Properties

Vermiculine shows inhibitory activities against Gram-positive bacteria, *Trypanosoma cruzi* and *Leishmania braziliensis*, but only very weak activity against yeasts or *Mycobacteria* was noted. The antibiotic activity of vermiculine against various microorganisms *in vitro* is shown in Table 2. Antibacterial and antifungal activitites were determined by the agar diffusion method. The activity against *T. cruzi* and *L. braziliensis* was tested using the method described by NEMEC *et al.*[2]

Table 2. Antimicrobial activity of vermiculine

Test microorganisms	Minimum inhibitory concentration (mcg/ml)
Bacillus subtilis SDPC 1 : 220	20
Bacillus subtilis BS-1	15
Staphylococcus pyogenes-aureus B-3	25
Staphylococcus aureus Sta-2	30
Sarcina lutea	25
Bacillus cereus	10
Bacterium linens	10
Mycobacterium bovis BCG	500
Candida pseudotropicalis	100
Trypanosoma cruzi	10
Leishmania braziliensis	0.5

Toxicity of vermiculine in mice was determinated by intraperitoneal injection with arabic gum. The intraperintoneal acute LD_{50} was found to be 420 mg/kg.

Discussion

At the present time only the production of the acid spolysaccharides luteic acid[4,5] and mucilate[6], have been described in *Penicillium vermiculatum* DANGEARD. Vermiculine is therefore the first biologically active metabolite isolated from this culture. It is not identical with any of the known antiprotozoal antibiotics, or with any of the fungal metabolites described to this day. By some of its chemical and physico- chemical properties, as well as by its antiprotozoal activity, it is similar, however, to ophiobollin A[7,8] and ophiobolosin A[9]. The structure of the vermiculine is now being studied in our laboratory.

The authors are grateful to Dr. V. LUKÁČOVÁ, Mrs. A. FUSKOVÁ, Dr. J. BALANOVÁ, M. MOLÍNKOVÁ and J. STROSSOVA for technical assistance throughout this work. They are also indebted to the members of the analytical laboratory of Lachema for the microanalysis and IR spectra.

References

1) NEMEC, P.; J. BALAN & L. EBRINGER : Antiprotozoal antibiotics. I. Method of specific screening. J. Antibiotics, Ser. A 16 : 155~156, 1963
2) NEMEC, P.; L. KRIŽKOVÁ, J. BALAN, J. BALANOVÁ & M. KUTKOVÁ : Incidence of antiprotozoal and antivermal antibiotics in fungi. I. Class fungi imperfecti. J. Antibiotics 22 : 345~350, 1969
3) FASSATIOVA, O. & V. HARTMANOVA : Find of *Talaromyces (Penicillium) vermiculatum*, DONG. BENJ. in mine in the Czechoslovakia. Ceská Mykologie 25 : 1971 (in press)

4) BIRKINSHAW, J. H. & H. RAISTRICK: Studies in the biochemistry of microorganisms. XXVII. The production of luteic acid from various sources of carbon by *Penicillium luteum* ZUKAL. Biochem. J. 27: 370~375, 1933
5) RAPER, K. B. & C. THOM: A manual of the Penicillia. p. 609, Hafner Publishing Co., New York, London, 1968
6) NAKAMURA, N.; J. OOYAMA & O. TANABE: Production of mucilate by mold. II. Taxonomic study of the mucilate producing *Penicillium* and production of the mucilate by known *Penicillium* species. J. Agr. Chem. Soc. Japan 36: 520~524, 1962
7) NAKAMURA, M. & K. ISHIBASHI: On the new antibiotic "ophiobolin", produced by *Ophiobolus miyabeanus*. J. Agr. Chem. Soc. Japan 32: 739~744, 1958
8) TSUDA, K.; S. NOZOE, M. MORISAKI, K. HIRAI, S. OKUDA, L. CANONICA, A. FICCHI, M. GALLI KIENLE & A. SCALA: Nomenclature of ophiobolins. Tetrahedron Letters 1967-35: 3369~3370, 1967
9) OHKAWA, T. & T. TAMURA: Studies on the metabolites of *Cochliobolus miyabeanus*. I. Ophiobolosin A and Ophiobolosin B. Agr. Biol. Chem. 30: 285~291, 1966