ANTIBIOTICS FROM BASIDIOMYCETES. VII[1]

CRINIPELLIN, A NEW ANTIBIOTIC FROM THE BASIDIOMYCETOUS FUNGUS *CRINIPELLIS STIPITARIA* (FR.) PAT.

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A crystalline antibiotic, which we have named crinipellin, was isolated from submerged cultures of the basidiomycete *Crinipellis stipitaria*, strain No. 7612. High resolution mass spectrometry yielded the formula $C_{22}H_{28}O_5$. The antibiotic is most active against Gram-positive bacteria, although yeasts and filamentous fungi are affected to a lesser extent. Crinipellin exhibits high *in vitro* inhibitory activity against the ascitic form of EHRLICH carcinoma. The incorporation of precursors of DNA-, RNA-, and protein syntheses in EHRLICH carcinoma (and in *Bacillus brevis*) cells was completely inhibited at 5(10) μg/ml. In *Bacillus brevis* the inhibition of the incorporation of uridine was found to be due to an interference by crinipellin with the transport of the precursor into the cells.

Crinipellis stipitaria is a small agaric growing on dead and – more preferentially – on living parts of grasses. Within the family Tricholomataceae the genus *Crinipellis* shows some morphological relationships to the genus *Marasmius*[2], but also to genera with cyphelloid fruiting bodies as previously shown by AGERER[3]. *Crinipellis stipitaria*, strain No. 7612, which was obtained from the spore-print of a specimen collected near Tübingen, was found to produce an antibacterial and antifungal metabolite. From the culture broth of the fungus we have isolated the antibiotic, which has been named crinipellin. This report deals with the fermentation, the isolation procedure, and the physico-chemical and biological properties of crinipellin.

The determination of the structure will be subject of a second publication.

Materials and Methods

Fermentation

Crinipellis stipitaria 7612 was maintained on YM-medium composed of (g/liter): yeast-extract 4, malt-extract 10, glucose 4. Mycelium from one agar slant was used to inoculate 500-ml flasks containing 150 ml of YM-medium. The seed flasks were incubated on a rotary shaker at 22°C for 10 days. Fermentation was carried out in a New Brunswick FS 314 fermentor containing 10 liters of YM-medium. After inoculation with 300 ml of seed cultures and addition of 2 ml Niax polyol antifoam the fermentation was conducted at 22°C for 8 days with agitation (150 rev/min) and aeration (3 liters/min). Antibiotic production was followed by paper-disc / agar-diffusion assay using *Bacillus brevis* as test organism.

Isolation

After removal of the mycelium by filtration, the filtrate (9 liters) was extracted twice with 2 liters each of ethyl acetate. The organic phase was concentrated furnishing 3 g of crude extract. This

was applied to 2 successive columns of silica gel (Serva, "Mallinckrodt"-silica gel, 100 mesh; 2.5× 20 cm), which were eluted with $CHCl_3$ – EtOH (99: 1), and for the second column $CHCl_3$, respectively. The fractions containing the antibiotic were pooled, the solvent evaporated and the residue chromatographed on Sephadex LH-20. Elution with methanol yielded the crude crinipellin, which was purified by chromatography on silica gel with cyclohexane – AcOEt (1: 1) as eluent. Recrystallization from MeOH resulted in 50 mg of colorless crystals of crinipellin.

Thin-layer chromatography (TLC)

TLC was performed on silica gel plates (Merck, 60 F_{254}). The spots of crinipellin were detected under ultraviolet light (254 nm) or by bioautography on agar plates seeded with *Bacillus brevis* spores.

Test organisms and antimicrobial spectrum

The fungi and *Aerobacter aerogenes, Arthrobacter citreus, Sarcina lutea* and *Streptomyces viridochromogenes* were grown on YM-medium, *Clostridium pasteurianum* on RCM-medium 5411 (Merck) and all other bacteria on nutrient broth (Difco). The incubation temperature was 27°C for *Arthrobacter citreus, Corynebacterium insidiosum, Micrococcus roseus, Pseudomonas fluorescens, Sarcina lutea, Streptomyces viridochromogenes* and all the fungi; the other bacteria were incubated at 37°C. The minimal inhibitory concentrations (MICs) of crinipellin were determined by the conventional serial broth dilution assay. The effect on the growth of *Clostridium pasteurianum* was measured with the plate diffusion test (30 μg antibiotic per paper disc).

Macromolecular syntheses in cells of the ascitic form of EHRLICH carcinoma (ECA)

Macromolecular syntheses in ECA cells grown in female mice were performed as described previously[4]. Cells were suspended in phosphate-buffered saline[5] (0.1 mg/ml (8.5 units) heparin and 1 mg/ml glucose) at a density of 3×10^6 cells/ml and preincubated with the antibiotic for 10 minutes at 37°C. To test the effect on DNA-, RNA-, or protein synthesis, 3 ml of the cell suspension were then incubated with 0.1 μCi (2-^{14}C)-thymidine (61 mCi/mmol), 0.1 μCi (2-^{14}C)-uridine (53 mCi/mmol) or 0.1 μCi (1-^{14}C)-leucine (59 mCi/mmol). After 20 minutes at 37°C the cells were centrifuged, suspended in 5% trichloroacetic acid (TCA) and collected on cellulose nitrate filters. The radioactivity was determined by liquid scintillation counting.

Measurement of respiration

Oxygen uptake from an oxygen-saturated suspension of *Bacillus brevis* in nutrient broth was measured polarographically with a Clark electrode.

Test for hemolytic activity

Hypotonic hemolysis (control) of human erythrocytes was determined by the hemiglobincyanide-method, Merckotest 3317 (Merck)[6]. The effect of crinipellin on erythrocytes was tested in a similar way using an isotonic medium.

Transport studies

For experiments on uridine uptake, cells of *Bacillus brevis* in nutrient broth were preincubated at 37°C for 10 minutes with or without antibiotic. Then 0.2 μCi (^{14}C)-uridine was added and at the times indicated two 0.5-ml samples of cell suspension were withdrawn: One was suspended in 2 ml 5% TCA and assayed for radioactivity in the acid-insoluble material, the other was quickly poured over a membrane filter, which had been soaked with ice-cold uridine solution (0.05% in H_2O), and washed with 5 ml of the uridine solution. After drying, the radioactivity retained on the filter was determined.

Results and Discussion

Isolation and Physico-chemical Properties

In the course of our screening, crinipellin was detected in the culture broth of all examined (7) strains of *Crinipellis stipitaria*, collected in Germany, Italy, and the U.S.A. The antibiotic could not be found in cultures of the related species *Crinipellis perniciosa* (STAHEL) SING.

Crinipellin, obtained as described in the experimental section, crystallizes from methanol as

colorless rectangular plates, melting point 107°C. It is readily soluble in benzene, chloroform, carbon tetrachloride, ethyl acetate, methanol, ethanol, slightly soluble in cyclohexane or petroleum ether, and is almost insoluble in water.

The chromatographic behavior of crinipellin on TLC is described in Table 1. The antibiotic shows positive color reactions with dichlorofluorescein (pink), $KMnO_4$, and CARR-PRICE and KAGI-MIESCHER reagents.

The optical rotation was measured in ethyl acetate: $[\alpha]_D^{22} - 136°$ (c 0.66).

Table 1. Chromatographic behavior of crinipellin

Solvent system	Rf-value
Cyclohexane – ethyl acetate – formic acid (120: 40: 5)	0.39
Benzene – acetone – acetic acid (70: 30: 5)	0.92
Chloroform	0.89

Fig. 1. IR-spectrum of crinipellin (KBr)

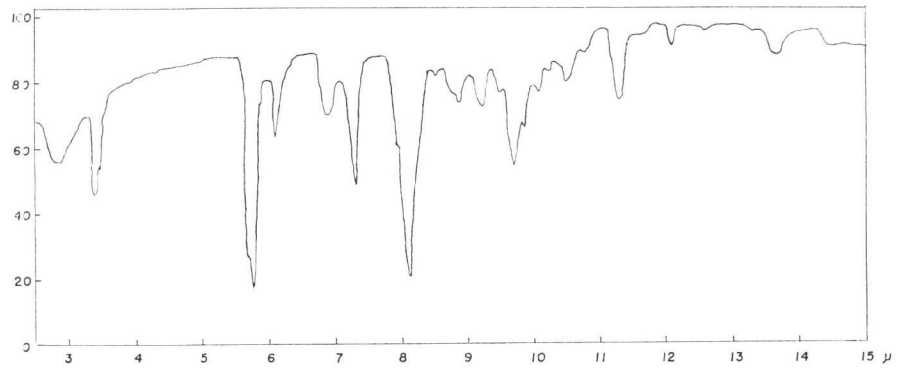
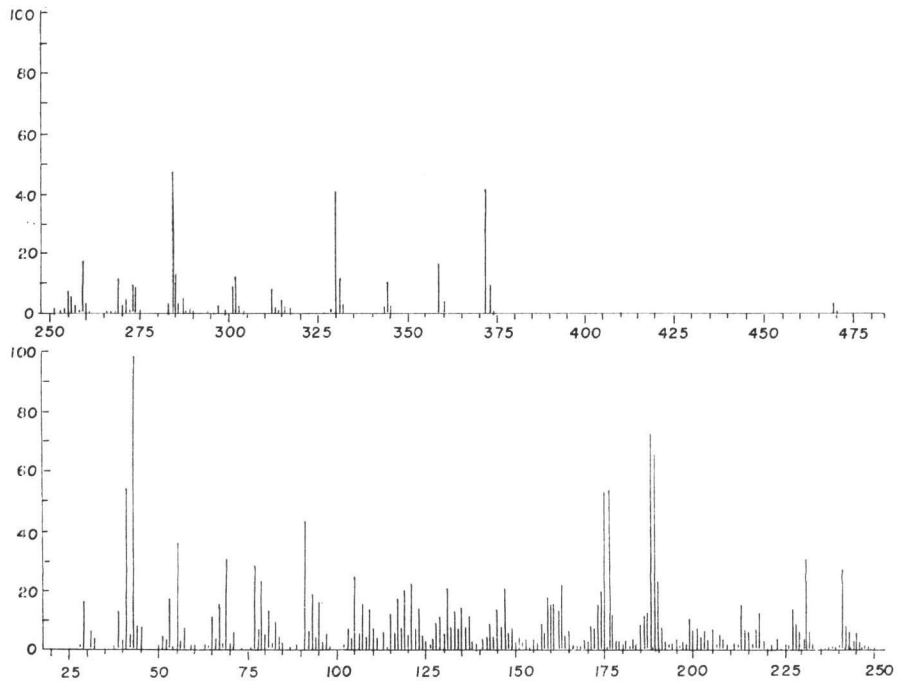


Fig. 2. Mass-spectrum of crinipellin (mass spectrum was determined on an A.E.I. MS 50 mass spectrometer; 70 eV, direct insertion, 180°C)



The UV-spectrum of crinipellin in methanol indicated the maximum at 234 nm (log $\epsilon = 3.74$), the IR-spectrum is shown in Fig. 1.

The molecular formula of crinipellin was confirmed to be $C_{22}H_{28}O_5$ by mass spectroscopy (mass spectrum, Fig. 2) and high resolution analysis of the molecular ion (*m/e* 372).

Biological Properties

Table 2 shows the minimal inhibitory concentrations of crinipellin against a variety of bacteria and fungi. With the exception of *Proteus vulgaris* the antibiotic was not active against Gram-negative bacteria, whereas Gram-positive bacteria were affected at concentrations averaging approximately 5 μg/ml. Up to 30 μg/ml no effect on the anaerobe *Clostridium pasteurianum* could be observed. Furthermore crinipellin proved to be active against yeasts and filamentous fungi.

Cellular DNA-, RNA-, and protein syntheses were examined in ECA cells by determining the incorporation of (^{14}C)-thymidine, (^{14}C)-uridine and (^{14}C)-leucine into the acid-insoluble fractions. The inhibitory effect was measured as the reduced incorporation of the appropriate precursor, as compared with the controls. As shown in Fig. 3, at low concentrations DNA- and RNA syntheses were preferentially inhibited; at 5 μg/ml all macromolecular syntheses were completely inhibited. Similar results could be obtained with exponentially growing cells of *Bacillus brevis* (data not shown).

Since no specific inhibition of one of the macromolecular syntheses could be observed, we

Table 2. Antimicrobial spectrum of crinipellin

		MIC (μg/ml)
Bacteria	Pseudomonadales	
	Pseudomonas fluorescens	>50
	Eubacteriales, Gram-negative	
	Aerobacter aerogenes	>50
	Escherichia coli	>50
	Proteus vulgaris	5
	Eubacteriales, Gram-positive	
	Arthrobacter citreus	5
	Bacillus brevis	2~5
	Bacillus subtilis	5
	Clostridium pasteurianum	>30
	Corynebacterium insidiosum	5
	Micrococcus roseus	5
	Mycobacterium phlei	5~50
	Sarcina lutea	2
	Staphylococcus aureus	5
	Actinomycetales	
	Streptomyces viridochromogenes	5
Fungi	Ascomycetes	
	Candida albicans	50
	Neurospora crassa	30
	Penicillium notatum	15~30
	Saccharomyces cerevisiae S 288c	30
	Saccharomyces cerevisiae FL200	>50
	Saccharomyces cerevisiae is 1	15
	Basidiomycetes	
	Rhodotorula glutinis	30

Fig. 3. Effect of crinipellin on macromolecular syntheses in EHRLICH carcinoma ascites cells in % of the control without antibiotic
(1) protein synthesis
(2) RNA synthesis
(3) DNA synthesis
Controls without antibiotic; incorporation per ml cell suspension: (^{14}C)-leucine 6887 cpm, (^{14}C)-uridine 10054 cpm, (^{14}C)-thymidine 1621 cpm.

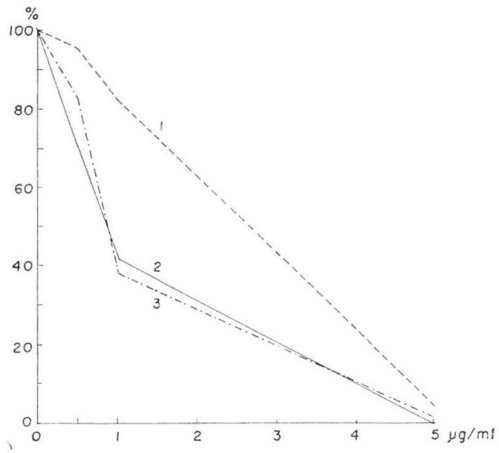


Fig. 4. Effect of crinipellin on the oxygen uptake of *Bacillus brevis* cells.

Respiration was measured polarographically in nutrient broth containing 10^8 cells/ml. Indicated by the arrow, different amounts of crinipellin were added.

(1) control without antibiotic
(2) 1 μg/ml
(3) 2.5 μg/ml
(4) 10 μg/ml

To examine the inhibitory effect of crinipellin on RNA synthesis, 0.1 μCi (^{14}C)-uridine was added to the same cell suspension together with crinipellin; after 5 minutes the effect on the incorporation of the precursor into TCA precipitable material was measured.

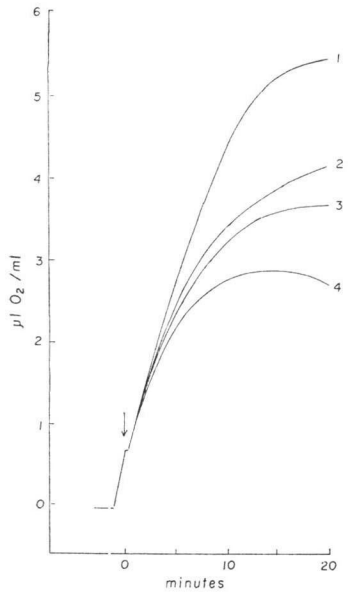
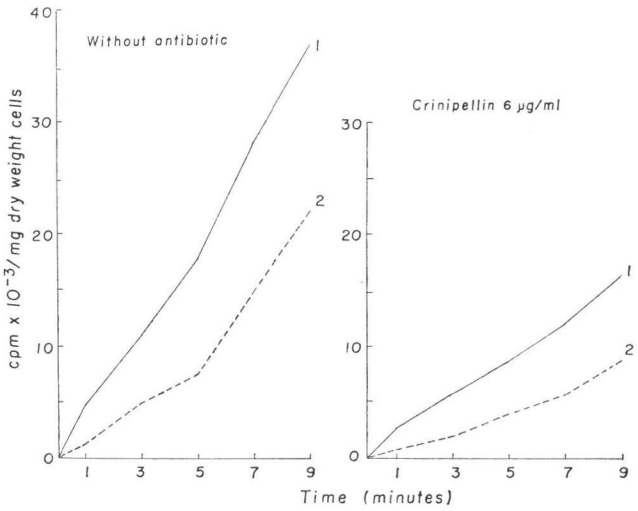


Fig. 5. Effect of crinipellin on uridine uptake and RNA synthesis in cells of *Bacillus brevis*

(1) Total uridine uptake
(2) Uridine incorporation into RNA



tested the effect of crinipellin on respiration and on uridine transport in intact cells of *Bacillus brevis* (Figs. 4 and 5). Fig. 4 shows the reduction in oxygen uptake by the bacterial cells as a function of the concentration of crinipellin. Five minutes after the addition of 10 μg/ml of crinipellin, respiration was reduced to 75% of the control; the incorporation of (^{14}C)-uridine however was much more affected (30% of the control).

In Fig. 5 the relation of uridine uptake and uridine incorporation into TCA-insoluble material with or without antibiotic is demonstrated. As shown, crinipellin reduced the uptake of the precursor into the cells, but did not alter the percentage of incorporated uridine, compared with the amount of uridine taken up: In the control after 9 minutes of incubation 59% of the radioactivity taken up by the cells could be found in the TCA-insoluble material, whereas in the presence of the antibiotic 54% of the uridine taken up was incorporated. (In the same test rifampicin did not inhibit uridine transport at a concentration of 5 μg/ml, but totally inhibited the incorporation of the precursor). Because of these findings we tend to assume that crinipellin may interact with components of the cytoplasmic membrane, which are involved in the transport of uridine and most likely other precursors.

In accordance with this assumption we found that in isolated nuclei of ECA cells the synthesis of RNA itself is not affected by concentrations of crinipellin comparable to those needed for complete inhibition of uridine uptake in whole cells (data not shown). Up to 100 μg/ml crinipellin did not cause hemolysis of human erythrocytes—in our opinion a detergent-like mode of action can therefore

be disregarded.

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