ANTIBIOTICS FROM BASIDIOMYCETES

XXXII.† STROBILURIN E: A NEW CYTOSTATIC AND ANTIFUNGAL (*E*)-β-METHOXYACRYLATE ANTIBIOTIC FROM *CREPIDOTUS FULVOTOMENTOSUS* PECK††

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Strobilurin E is a novel antibiotic of the (*E*)-β-methoxyacrylate (MOA) class produced by mycelial cultures of the agaric *Crepidotus fulvotomentosus*. In addition to an inhibition of fungal respiration, a feature of all MOA-antibiotics, the compound exhibits very high cytostatic activities which are accompanied by reversible morphological alterations of the cells.

The strobilurins, oudemansins and related synthetic mimics constitute a new class of respiration inhibitors which inhibit the electron transfer in the bc_1 complex. These compounds, both natural and synthetic, contain an (*E*)-β-methoxyacrylate (MOA) moiety which is responsible for the antifungal and respiration inhibiting properties[2]. Strobilurin E was detected and isolated in the course of a screening program for new fungal metabolites capable of reversibly inducing morphological alterations of tumor cells[3].

Materials and Methods

Crepidotus fulvotomentosus Peck, Strain 80145

The producing strain was derived from a fruiting body collected in North America. The specimen showed the characteristics of the genus and species[4]. The strain is deposited in the culture collection of the Lehrbereich Biotechnologie of the University of Kaiserslautern.

Fermentation

For maintenance on agar slants the fungus was grown in YMG medium composed of: Yeast extract 0.4%, malt extract 1%, glucose 0.4%, agar 2%. Fermentations were carried out in a Biolafitte C-6 fermenter in 20-liter of a medium containing in 1 liter: Glucose 20 g, peptone 5 g, yeast extract 5 g, KH_2PO_4 0.5 g, $MgSO_4 \cdot H_2O$ 1 g, $FeCl_3$ 10 mg, $ZnSO_4$ 1.78 mg and $CaCl_2$ 73.5 mg. The fermenter was incubated at 22°C with aeration (1.4 liters air/minute) and agitation (200 rpm).

Isolation

After three weeks of fermentation strobilurin E was extracted from the lyophilized mycelia (271 g) with 6-liter of methanol. Evaporation of the organic phase yielded a crude extract (98 g) which was applied

† See ref 1.

†† Dedicated to Prof. Dr. HANS ZÄHNER on occasion of his 60th birthday.

onto a flash column containing silica gel (Merck 60; 5 × 10 cm). After elution with toluene - ethyl acetate (9 : 1) 609 mg of an enriched product was obtained. This was further purified by preparative medium pressure liquid chromatography on C-18 silica gel (Merck 40; 2.5 × 25 cm; elution with MeOH - H_2O, 8 : 2) and by HPLC (silica gel, Merck 7; 2.5 × 25; elution with cyclohexane - propanol, 8 : 2). 79 mg of strobilurin E were obtained from 20-liter of culture.

The structure of strobilurin E (**1**) was determined by spectroscopic methods and comparison with synthetic model compounds[5].

Biological Assays

The assays for antimicrobial activity were performed as described previously[6].

Effect of Strobilurin E on Protein, RNA and DNA Syntheses of ECA and HeLa S3 Cells

Cells of the ascitic form of Ehrlich carcinoma (ECA) and HeLa S3 cells were grown in suspension culture in F-12 medium containing 15% horse serum (ECA) or 10% fetal calf serum (HeLa) at 37°C for 2 days. After centrifugation (100 × *g*) the cells were washed twice with and suspended ($1 \sim 1.5 \cdot 10^6$ cells/ml) in phosphate buffered saline (PBS) containing Ca^{2+} (1.1 mM), Mg^{2+} (0.6 mM), and glucose where indicated. The cells (1×10^6/ml) were preincubated with the antibiotics for 30 minutes at 37°C and then transferred to test tubes containing the precursors. The incorporations of radiolabelled thymidine, uridine and leucine into DNA, RNA and protein, respectively, were determined as described previously[7].

Effect of Strobilurin E (**1**) and Strobilurin A (**2**) on the Growth of HeLa S3 Cells

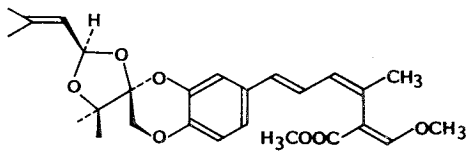
Growth of cells was measured as proportional to the increase of protein content. The values are given as the percentage of the controls. HeLa S3 cells were grown as monolayer cultures in F-12 medium containing 10% fetal calf serum at 37°C in microtiter plates in a humidified atmosphere. After 24 hours the medium was removed from the cells and replaced by the same medium containing the antibiotics. After 3 days the cells were examined under the microscope. To determine the protein contents the cells were washed with PBS, lysed with 1% of Triton X-100, treated with bicinchonic acid (BCA)-reagent (Pierce) and the resulting blue complex was measured in a multiphotometer (BIO-RAD)[8].

Effect of Strobilurin E on the Proliferation of HeLa S3 Cells

HeLa S3 cells were grown in microtiter plates with 24 wells as described above. The cells were incubated for three days with strobilurin E or daunorubicin as a control. After 1, 2, and 3 days, the cells were incubated with 0.1 μCi [^{14}C]thymidine for 24 hours. After suspending the cells in PBS containing 0.9% trypsin and 0.02% EDTA the incorporation of radioactivity into trichloroacetic acid precipitable material (DNA) was measured. In a parallel experiment, after 3 days the cells were washed twice, fresh medium without strobilurin E was added, and the incorporation of thymidine determined.

Effect of Strobilurin E on the Cellular ATP-content, Growth and Respiration of HeLa S3 Cells and *Penicillium notatum*

HeLa S3 cells were grown as described above. The cells were incubated for a maximum of 3 days with the test compounds. The ATP-content was measured with the luciferin-luciferase reaction (Bioluminescent Somatic Cell Assay Kit, No FL-ASC, Sigma). The cells of one well (diameter 1 cm) were incubated with 300 μl of "somatic cell ATP releasing reagent". After 15 minutes the cells were centrifuged (1 minute, 10,000 × *g*) and the supernatant was mixed with 200 μl enzyme solution ("ATP assay mix"). The emitted light was measured immediately with a fluorescence spectrophotometer (emission wavelength: 550 nm). The protein content was measured as described above. The inhibition of respiration of HeLa S3



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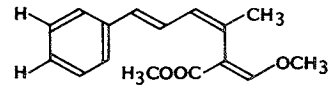

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cells (1×10^8/ml) and *P. notatum* (25 mg/ml) were measured using a Clark-type oxygen microelectrode in a closed vessel of 2.5 ml volume. The inhibitors were added in ethanol.

Effect of Strobilurin E on the Multiplication of Vesicular Stomatitis Virus (VSV) in Baby Hamster Kidney Cells (BHK-21)

VSV (ATCC VR 158, Indiana strain) was propagated in BHK cells (BHK-21; ATCC CCL 10) grown in microtiter plates. BHK-21 cells were grown in modified EAGLE's BME medium containing 10% Tryptose phosphate broth and 10% fetal calf serum. After the removal of the medium from the cells 250 plaque forming units (PFU) of VSV in 25 μl of modified EAGLE's BME medium with 2% of fetal calf serum were added and incubated for 1 hour at 37°C. Then 75 μl of the same medium containing the compounds to be tested were added and the cultures incubated until 90% of the cells in the controls containing no antibiotics were lysed as counted in the microscope. The effect of the antibiotics on virus multiplication was measured by estimation of the virus titer. After withdrawing 10 μl aliquots the number of plaques formed on BHK-21 cells were determined with suitable dilution of the virus-containing samples.

Results and Discussion

Strobilurin E (**1**) exhibits strong antifungal activities which compare favorably with those of strobilurins A, B and oudemansins[6,9]. In the plate diffusion assay (Table 1) *Mucor miehei* and other filamentous fungi were strongly inhibited by 0.1 ~ 1 μg of strobilurin E. Respiration of *P. notatum* was completely blocked by 30 ng/ml of strobilurin E. No antibacterial activity was detected[3].

The most conspicuous activity of strobilurin E is its inhibitory action on the growth of human tumor cells (HeLa S3) starting at 2 ng/ml (Fig. 1). Up to a concentration of 25 μg/ml strobilurin E did not lead to cell lysis and the cells still showed a residual growth as measured by increase in protein-content. Fig. 2 shows the effect of strobilurin E on the proliferation (thymidine incorporation) of HeLa S3 cells as a function of time. Cellular proliferation was not completely blocked by up to concentrations of 250 ng/ml of strobilurin E. Cells preincubated with 10 ~ 25 ng/ml strobilurin E resumed growth after the removal of the antibiotic. It is thus concluded that strobilurin E exhibits high cytostatic activities which are partially reversible. Irreversible toxic effects

Table 1. Antifungal activity of strobilurin E in the agar diffusion assay.

Organism	Diameter of inhibition zone (mm)	
	0.1[a]	1[a]
Absidia glauca	8	10
Alternaria porri	16	20
Aspergillus ochraceus	8	15
Botrytis cinerea	—	20
Candida albicans	—	12
Cladosporium cladosporioides	15	20
Curvularia lunata	13	15
Epicoccum purpurascens	12	15
Mucor miehei	13	18
Nematospora coryli	16	23
Neurospora crassa	20	25
Paecilomyces varioti	—	9
Penicillium islandicum	—	15
P. notatum	10	18
Phoma clematidina	10	20
Phytophthora infestans	—	10
Rhodotorula glutinis	—	13

[a] μg/disc.
—: No inhibition zone.

Fig. 1. Effect of strobilurins E and A on the growth of HeLa S3 cells.

● Strobilurin E, ■ strobilurin A.
Protein increase in the controls (100%) was 12 μg/ml in 3 days.

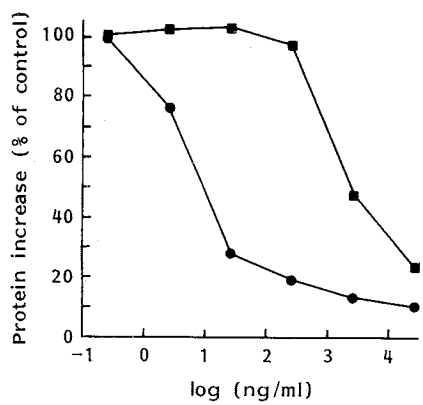


Fig. 2. Effect of strobilurin E on the proliferation of HeLa S3 cells.

Controls without antibiotics (100%): 28.380~36.850 cpm. strobilurin E: ● 10 ng/ml, ■ 25 ng/ml, ▲ 50 ng/ml, □ 75 ng/ml, ▼ 250 ng/ml. daunorubicin: ○ 100 ng/ml, —— medium replacement.

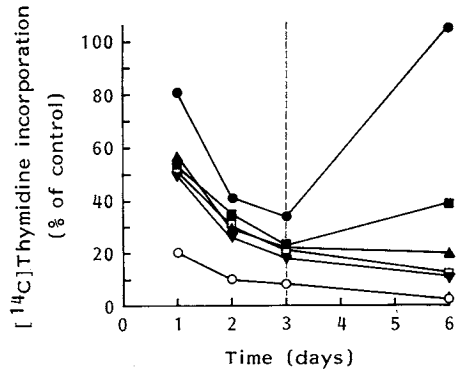


Fig. 3. Effect of strobilurin E on the cellular ATP-content and growth of HeLa S3 cells.

ATP-content of the controls: 35.1~46.3 (nmol/mg cell protein). ▲ ATP-content: strobilurin E (1 μg/ml). ■ ATP-content: strobilurin E (1 μg/ml)+2-deoxy-D-glucose (10 mM). ○ protein-content of control. △ protein-content: strobilurin E (1 μg/ml).

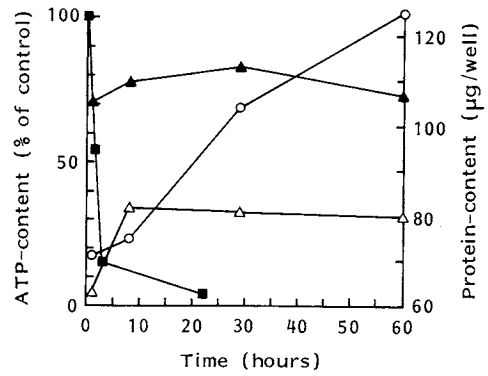


Table 2. Effect of strobilurin E on protein, RNA, and DNA syntheses of ECA (A) and HeLa S3 (B) cells.

(A)

Strobilurin E (μg/ml)	Glucose (μg/ml)	Incorporation (cpm) of precursor		
		Leucine	Uridine	Thymidine
0	0	53,296	19,540	5,022
1	0	1,066	391	351
0	100	55,854	20,168	5,156
1	100	45,242	16,134	4,743
10	100	46,359	17,748	4,949

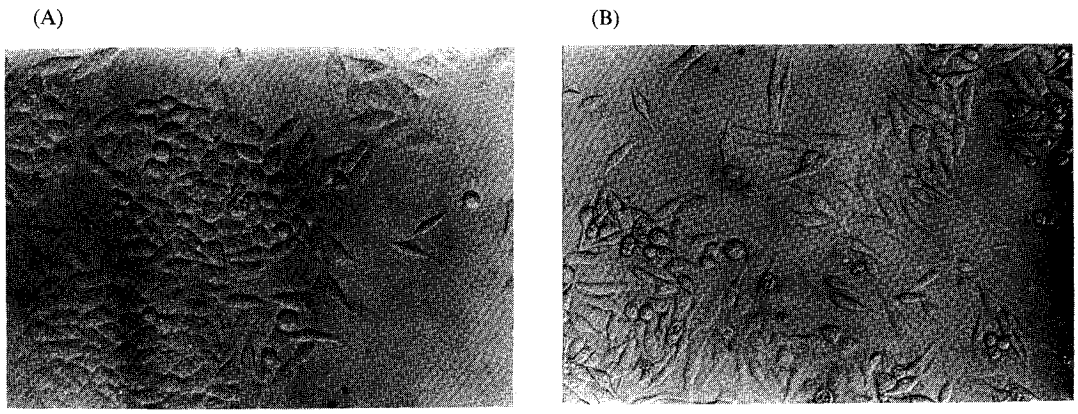
(B)

Strobilurin E (μg/ml)	Glucose (μg/ml)	Incorporation (cpm) of precursor		
		Leucine	Uridine	Thymidine
0	0	18,775	9,406	2,656
1	0	14,112	5,321	2,630
10	0	13,893	4,797	2,701
0	100	29,389	7,069	1,748
1	100	18,320	6,450	1,653
10	100	17,633	3,958	1,485

(lysis) were observed only at strobilurin E concentrations above 50 μg/ml. When compared to strobilurin E, strobilurin A (**2**)[6] possesses lower cytostatic activities. The growth of HeLa S3 cells is reduced to 50% at approximately 100 times higher antibiotic concentrations (1~2 μg/ml).

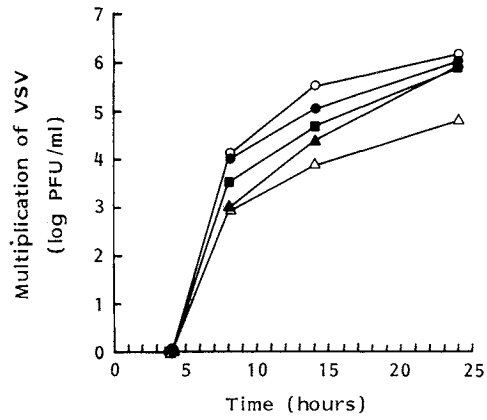
The effect of strobilurin E on the syntheses of macromolecules in cells of the ascites form of ECA is shown in Table 2A. Similar to oudemansins[10] and other MOA-derivatives the compound strongly inhibited protein, RNA and DNA syntheses which were reversed by the addition of glucose. The inhibition of respiration (at 1 μg/ml) led to a depletion of the cellular ATP-pool which was restored by glycolysis in the presence of glucose.

Fig. 4. Morphological differentiation of HeLa S3 cells in the presence of strobilurin E.
(A) Normal epithelial monolayer (control). (B) Cells in 25 ng/ml strobilurin E.



In contrast to the effect of strobilurin E on ECA cells, HeLa S3 cells responded different to the antibiotic. As shown in Table 2B the macromolecular syntheses in HeLa S3 cells were inhibited to a much lesser extent and this inhibition was not reversed by the addition of glucose. Since respiration of HeLa S3 cells was completely blocked at concentrations above 60 ng/ml these results are indicative of an endogenous pool of reserve carbohydrates which is sufficient to meet short time energy demands by glycolysis.

Fig. 5. Effect of strobilurin E on the multiplication of VSV in BHK-21 cells.



The complete inhibition of oxidative phosphorylation by strobilurin E in glucose-containing F-12 medium resulted in a 30% drop of the HeLa S3 cell's ATP-content within 10 hours (Fig. 3). This level was not sufficient for growth and proliferation but apparently allowed the cells to survive for some days. 2-Deoxy-D-glucose which effectively blocked ATP-production by glycolysis completely depleted the cellular ATP-pool.

Concomitant with the inhibition of respiration and the reduction of cellular ATP-content, HeLa S3 cells showed an interesting alteration from an epithelial (Fig. 4A) to a fibroblast-like morphology (Fig. 4B). The morphological alteration was observed with strobilurin E (25 ng/ml) and strobilurin A (2,500 ng/ml) and was completely reversed after the removal of the antibiotics by several washings of the cells.

Fig. 5 shows the effect of strobilurin E on the propagation of VSV in BHK-21 cells. Multiplication of the virus was significantly reduced by concentration as low as 10 ng/ml. The presence of 25 ng/ml and 2.5 μg/ml of strobilurin E reduced the growth of BHK-21 cells to 39 and 28% of the controls without antibiotic.

Of the (*E*)-β-methoxyacrylate antibiotics isolated so far, strobilurin E exhibits the most potent and reversible cytostatic activities on mammalian cells. These are accompanied by a decrease of the cell's ATP-content and a change in morphology. The antiviral effects of strobilurin E on VSV multiplication

in BHK-21 cells are considered to result from the arrest of the host cell's metabolism.

Acknowledgments

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