THE STROBILURINS — NEW ANTIFUNGAL ANTIBIOTICS FROM THE BASIDIOMYCETE *STROBILURUS TENACELLUS* (PERS. ex FR.) SING.

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The strobilurins are two antifungal antibiotics which were isolated from the mycelium of *Strobilurus tenacellus* strain No. 21602. The strobilurins A and B are highly active against yeasts and filamentous fungi. *In vitro* antitumor activity was tested using cells of the ascitic form of EHRLICH carcinoma. The strobilurins strongly inhibited the incorporation of radioactive leucine, uridine, and thymidine into the acid-insoluble fraction of cells (protein, RNA, and DNA). The molecular formulas as determined by high resolution mass spectrometry are $C_{16}H_{18}O_3$ for strobilurin A and $C_{17}H_{19}ClO_4$ for strobilurin B.

The basidiomycete *Strobilurus tenacellus* is a small agaric growing on decaying cones of *Pinus silverstris*. Cultures of this species growing on agar plates or in submerged culture show marked antifungal activity. From the mycelium of *Strobilurus tenacellus* strain No. 21602 we have isolated two antibiotics which have been named strobilurins A and B. In the following paper we wish to report the fermentative production, the isolation, and the chemical and biological characterization of the strobilurins. The determination of the structure will be subject of a second publication.

Fermentation

Strobilurus tenacellus 21602 was maintained on agar slants of a yeast extract-malt extract (YM) medium (4 g yeast extract, 4 g glucose, 10 g malt extract per liter). For submerged cultivation a 150 ml of YM medium was inoculated with mycelium from one agar slant and incubated for 5 days on a rotary shaker at 22°C and 120 rpm. This culture was used to inoculate 10 liters of the same medium in a New Brunswick FS 314 fermentation apparatus. One ml of polyol antifoam was added initially, and the mycelia were grown at 22°C with mechanical stirring (150 rpm), and an aeration rate of 2 liters air/min.

Isolation

The mycelia from a 10-liter culture grown for 4 days were collected on a Büchner funnel, and washed several times with water. The cells (wet weight 210 g) were extracted first with 600 ml of methanol - acetone (2: 1), and then with 700 ml of methanol. The combined extracts were evaporated and the antibiotics were extracted with 100 ml of chloroform from the residue. The chloroform was dried over anhydrous Na_2SO_4 and evaporated yielding 3.1 g of a dark brown oil. The crude extract was

applied to a column (2.5 × 16 cm) with silica gel (Mallinckrodt), and eluted with chloroform (kept over aluminum oxide, activity I). The fractions containing strobilurin A, which was eluted first, or strobilurin B were pooled and the solvent evaporated. The crude strobilurins A (164 mg) and B (77 mg) were each dissolved in methanol and given on two columns (2.5 × 33 cm) with Sephadex LH-20, and eluted with the same solvent. From the fractions containing the antibiotic activities strobilurin A was obtained as a colorless oil (30 mg) and strobilurin B as colorless crystals (27 mg; after recrystallization from ethanol).

Physico-chemical Properties

Strobilurin A obtained as described is a colorless homogeneous oil. Strobilurin B is a colorless, crystalline antibiotic with a melting point of 95°C. The strobilurins are soluble in methanol, ethanol, acetone, ethylacetate, chloroform, and carbon tetrachloride, but are very poorly soluble in water. The strobilurins are optically inactive. The UV- spectrum of strobilurin A in ethanol shows maxima at 230 nm (ε 16,900), 237 nm (ε 15,330), and 294 nm (ε 21,850) whereas the maxima of strobilurin B are at 229 nm (ε 26,800) and 304 nm (ε 28,400). The chromatographic behaviour of the strobilurins on thin-layer chromatography is reported in Table 1. They give positive reactions with $KMnO_4$ and conc. sulfuric acid. Figs. 1 and 2 show the IR-spectra of the strobilurins. Mass spectrometry (A.E.I. MS 50 mass spectrometer, 70 eV, direct insertion, 150°) and high resolution of the molecular ions *m/e* 258 and 322 yielded the formulas $C_{16}H_{18}O_3$ for strobilurin A and $C_{17}H_{19}ClO_4$ for strobilurin B.

Biological Properties

Table 2 shows the antimicrobial spectra of the strobilurins in the serial dilution test and the agar plate diffusion test. They are highly active against filamentous fungi and yeasts, whereas bacteria are not affected by concentrations up to 20 μg/ml. *Arthrobacter citreus, Sarcina lutea,* and *Streptomyces viridochromogenes* were grown on YM medium and the other bacteria on nutrient broth (Difco). Conidia of filamentous fungi were seeded in malt agar (MA, 20 g malt extract, 20 g agar per liter) and yeasts were grown in yeast nitrogen base (Difco) containing 0.4% glucose and 2% agar. Fig. 3 shows the effect of the strobilurins A and B on the growth of *Botrytis cinerea* and *Rhizoctonia solani.* A small inoculum was placed in the center of petri dishes with MA containing no (control), 1 and 10 μg/ml antibiotic and each day the diameter of the growth zone was measured. When tested the same way the growth of *Pythium debaryanum* on corn meal agar containing 10 μg/ml strobilurin A or B was 37% of the control. The effect of the strobilurins on macromolecular synthesis in cells of the ascitic

Table 1. Thin-layer chromatographic behavior of the strobilurins

TLC was performed on Merck silica gel plates and the spots were detected by spraying with conc. H_2SO_4 or by bioautography on agar plates seeded with *Penicillium notatum* spores.

Solvent system	Rf	
	Strobilurin	
	A	B
Cyclohexane - ethylacetate - formic acid (120: 40: 5)	0.68	0.54
Benzene	0.22	0.18
Chloroform	0.72	0.65

Fig. 1. IR-Spectrum of strobilurin A (pure oil).

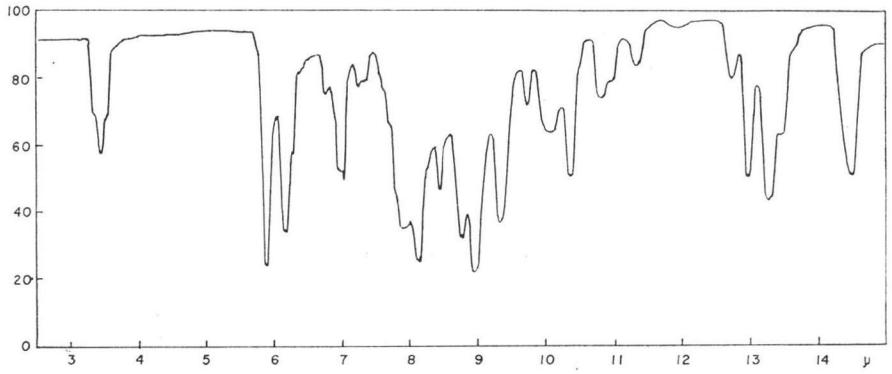

Fig. 2. IR-Spectrum of strobilurin B in KBr.

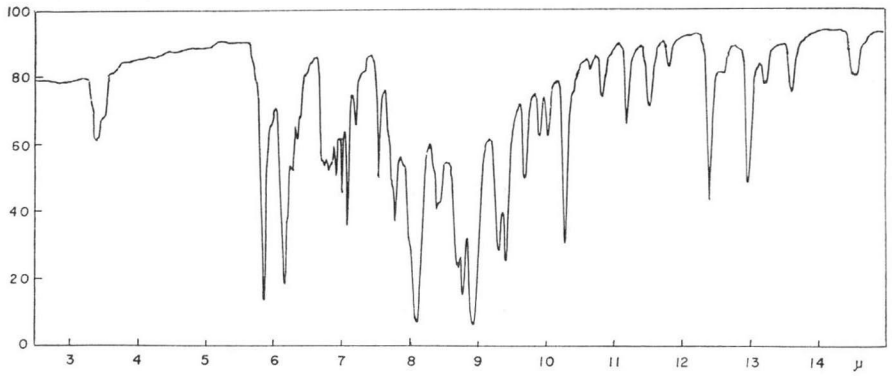

Fig. 3. Effect of strobilurins A and B on the growth of *Botrytis cinerea* and *Rhizoctonia solani*. Control without antibiotic (1); growth on agar plates containing 1 μg/ml strobilurin A (2) or B (3); growth on plates containing 10 μg/ml strobilurin B (4) or A (5).

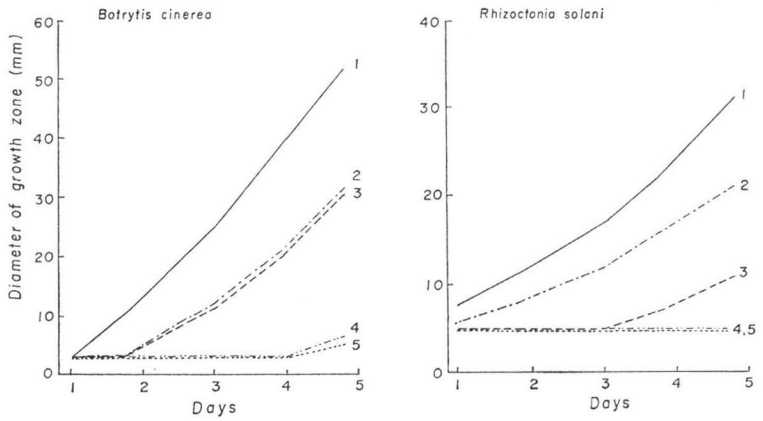

Table 2. Antimicrobial spectra of the strobilurins

Test organism	MIC (μg/ml), Strobilurin		
	A	B	
Aerobacter aerogenes	>20	>20	Serial dilution test
Arthrobacter citreus	>20	>20	
Bacillus brevis	>20	>20	
Bacillus subtilis	>20	>20	
Escherichia coli K12	>20	>20	
Leuconostoc mesenteroides	>20	>20	
Micrococcus roseus	>20	>20	
Proteus vulgaris	>20	>20	
Sarcina lutea	>20	>20	
Staphylococcus aureus	>20	>20	
Streptomyces viridochromogenes	>20	>20	
Aspergillus panamensis	22/28	18/22	Plate diffusion test diameter inhibition zone*
Candida albicans	30/38	22/29	
Fusarium cubense	—/—+	—/—	
Neurospora crassa	—/—	—/—	
Paecilomyces varioti	10/15	10/15	
Penicillium notatum	20/29	20/29	
Rhodotorula glutinis	30/35	30/35	
Saccharomyces cerevisiae	—/—	—/—	
Saccharomyces cerevisiae is 1	43/56	34/54	

* The figures before the slash refer to the size of the inhibition zone (mm) with discs receiving 1 μg strobilurin A or B; the figures after the slash refer to the inhibition zone with 10 μg antibiotic per disc.

\+ no inhibition.

form of EHRLICH carcinoma was tested in a similar way as described by WEITZEL *et al.*[1] The cells (3×10^6) in phosphate buffered saline[2] containing 51 units (0.6 mg) heparin (Serva) were preincubated with the antibiotics for 10 minutes at 37°C. The cell suspension was then transferred to test tubes containing 0.1 μCi (2-^{14}C)-uridine (53 mCi/mmol), 0.1 μCi L-(1-^{14}C)-leucine (59 mCi/mmol), or 0.1 μCi (2-^{14}C)-thymidine (61 mCi/mmol), and incubated at 37°C with gentle shaking. After 20 minutes the cells were centrifuged, the pellet suspended in 5% TCA, and the acid-insoluble material was collected on membrane filters. The radioactivity was determined by liquid scintillation counting. As shown in Table 3 the strobilurins A and B strongly inhibit protein, RNA, and DNA synthesis at concentrations as low as 0.2 μg/ml.

Table 3. Effect of the strobilurins A and B on DNA, RNA, and protein syntheses of EHRLICH carcinoma ascites cells

	Strobilurin added (μg/ml)	Incorporation of radioactivity (cpm) precursor		
		Thymidine	Uridine	Leucine
Control	0	3,547	18,714	21,913
Strobilurin A	0.2	570	283	1,330
	1.0	37	58	239
Strobilurin B	0.2	714	304	1,676
	1.0	53	57	255

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