STROBILURINS F, G AND H, THREE NEW ANTIFUNGAL METABOLITES FROM *BOLINEA LUTEA*

I. FERMENTATION, ISOLATION AND BIOLOGICAL ACTIVITY

ANDREAS FREDENHAGEN, ANTON KUHN and HEINRICH H. PETER*

Pharmaceutical Research Department, Biotechnology Subdivision, Ciba-Geigy Ltd., 4002 Basel, Switzerland

VINCENZO CUOMO and UGO GIULIANO

Division of Fermentation Products, Ciba-Geigy Ltd., Torre Annunziata, Italy

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Three new strobilurins F, G and H, antibiotics with antifungal activity, were isolated from cultures of *Bolinea lutea* Sacc. These new compounds differ from previously described analogs in their aromatic substitution. An HPLC method allows complete separation of all the components.

The strobilurins A, B[1,2], C[3] and E[4] as well as 4-methoxy-mucidin[5] and hydroxystrobilurin D[6] constitute a group of antifungal antibiotics (Scheme 1). The biological activity of these ubihydroquinone cytochrome C reductase inhibitors[7] depends on the presence of a terminal (*E*)-β-methoxyacrylate moiety[8]. The continuous interest in this structural type, mainly for agrochemical use, is documented in more than ten recent patents[9~12].

In the course of our screening program we found a strain of the microorganism *Bolinea lutea* Sacc. which produced three new strobilurins F, G and H in addition to the known antibiotics strobilurins A and B. In the following we describe the fermentation of the producing organism in fermenters and in shake flask and the isolation and HPLC analysis of the new products.

Materials and Methods

Microorganism

The microorganism was identified by its habitus and microscopic features as being most probable *B. lutea* Sacc.†. To our knowledge this is the first example of a strobilurin producing Ascomycetes species. Several Basidiomycetes have been described as strobilurin producers, namely *Strobilurus tenacellus*, *Oudemansiella mucida*, *Xerula logipes*, *Xerula melanotrichia*, *Mycena sanguinolenta* and a *Cyphellopsis* species.

Fermentation

B. lutea Sacc. was preserved as a frozen culture. For maintenance on agar slants and for seed cultures, the fungus was grown on a medium consisting of malt extract 1%, yeast extract 0.4%, glucose 0.4% and oatmeal 2%. To prepare the first vegetative inoculum, 100 ml of medium in a 500-ml Erlenmeyer flask was inoculated with the mycelium of one slant and incubated for 3 days on a rotary shaker at 25°C and 150 rpm. The second vegetative inoculum was prepared in the same way and seeded with an inoculum of 5%.

Ten liters of fermentation culture were inoculated with 5% of seed cultures. The medium (glucose

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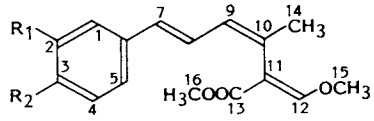
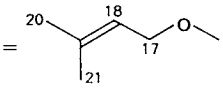
Scheme 1. Chemical structure of some strobilurins and compound **8**.

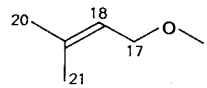


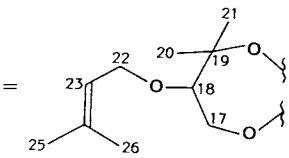
Strobilurin A (**1**) $R_1=R_2=H$

Strobilurin B (**2**) $R_1=OCH_3$ $R_2=Cl$

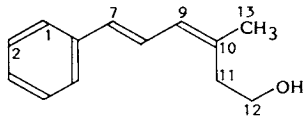
Strobilurin C (**3**) $R_1=$  $R_2=H$

Methoxymucidin (**4**) $R_1=H$ $R_2=OCH_3$

Strobilurin F (**5**) $R_1=OH$ $R_2=$ 

Strobilurin G (**6**) $R_1=R_2=$ 

Strobilurin H (**7**) $R_1=OCH_3$ $R_2=H$



8

30 g, maltose 10 g, oatmeal 20 g and yeast extract 4 g per liter) was adjusted to pH 7.5 before sterilization. The culture was incubated for 10 days at 25°C in a 20 liters fermenter (LSM Biolafitte; aeration 1 liter/minute; 150 rpm). Fermentation on a 4,000 liters scale was run accordingly.

For shake flask experiments (Fig. 2) 100 ml of fermentation medium in a 250-ml Erlenmeyer flask with one baffle were inoculated with 5% of the first vegetative culture and incubated during the appropriate time periods at 25°C and 150 rpm.

Isolation

General Remarks: MP's are uncorrected. Large scale liquid chromatography was done using a medium pressure system equipped with a Büchi pump B-681, Büchi glass columns B-685 and a Shimadzu UV-120-02 detector (0.5 mm pathlength). For HPLC a Hewlett Packard 1082A with a Kontron Uvikon 725 detector was used. Columns: Semipreparative: Silica gel Lichrosorb Si60, 5 μm; 16 × 250 mm; analytical: Silica gel Lichrosorb Si60, 5 μm; 4.6 × 250 mm.

To the whole culture broth (4,000 liters) a mixture of MeOH - EtOAc, 60 : 40 (4,800 liters) was added. Filtration was achieved using a filter aid and the filtrate was concentrated *in vacuo*. The concentrate was extracted once with 700 liters of $CHCl_3$ and the organic solvent removed *in vacuo*. The resulting brown oil was taken up in acetonitrile (*ca.* 10 liters) and extracted three times with half of the volume of cyclohexane. Evaporation of the acetonitrile-phase yielded 1.4 kg of an amorphous solid.

Crystallization of 1.27 kg of this material from ethanol - water (9 : 1) gave 308 g of strobilurin B (mp 97.5~99°C after recrystallization from ethanol[2]: 95°C). Polar impurities in the concentrated mother liquor were removed on a silica gel column (LiChroprep Si60, 25~40 μm; 1.8 liters; heptane - ethyl acetate, 80 : 20; 3 runs). The orange oil obtained (450 g) was separated into five fractions by column chromatography (same column as above; 10 runs; heptane - *tert*-butylmethyl ether, 85 : 15; last fraction eluted with heptane - EtOAc, 80 : 20; 100 ml/minute) which were combined according to UV trace (330 nm) and HPLC (see below):

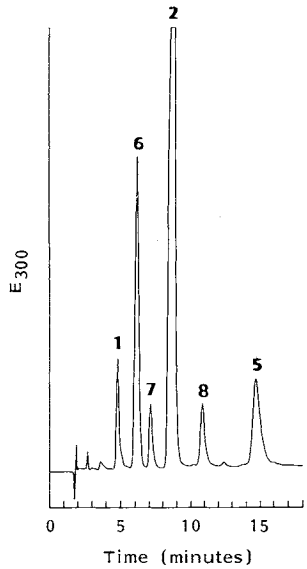
Fraction 1 (30 g) was crystallized from a very small amount of heptane at −20°C to give 12 g of drimenol (mp 94°C[13]: 97~98°C). The mother liquor contained mainly strobilurin A (18.4 g; content determined by HPLC: 80%). Fraction 2: 14.3 g of **6** as a yellowish oil. Fraction 3 (54 g) a part of which (21 g) was rechromatographed (LiChroprep Si60, 15~25 μm; 870 ml; heptane - *tert*-butylmethyl ether, 85:15; 2 runs; 45 ml/minute) to give **7** (1.1 g, 82% purity; analytical pure material obtained by semipreparative HPLC as a yellowish oil: 75 mg/run; hexane - *tert*-butymethyl ether, 80:20; 8.5 ml/minute; 320 nm; Rt 21 minutes) and **6** (2.9 g). Fraction 4 (48 g) was strobilurin B. Fraction 5 (50 g) was separated on silica gel (LiChroprep Si60, 25~40 μm; 1.8 liters; heptane - *tert*-butylmethyl ether, 80:20) to yield 17.5 g of **7** (85% pure; analytical pure sample by semipreparative HPLC as a yellowish oil: 257 mg/run; hexane - *tert*-butylmethyl ether, 80:20; 9 ml/minute; 333 nm; Rt 22 minutes) and 11 g of **5** after recrystallization from diethyl ether - hexane as colorless cubic crystals (mp 77.5~78°C).

HPLC Analysis

MeOH (10 ml) was added to a sample (10 ml) of the broth from a shake flask. After centrifugation the clear solution was concentrated with a Speedvac evaporator and distributed between EtOAc - hexane, 1:1 (10 ml) and water (2 ml). 20 μl of the organic phase were injected into the HPLC.

Quantitative distribution of compounds **1**, **2** and **5** to **8** was determined from HPLC elution curves on a silica column (conditions see Fig. 1). A typical time course in shake flasks is shown in Fig. 2.

Fig. 1. HPLC trace of the $CHCl_3$ extract of the whole broth after 4,000 liters fermentation, distribution between acetonitrile and cyclohexane and solvent evaporation.

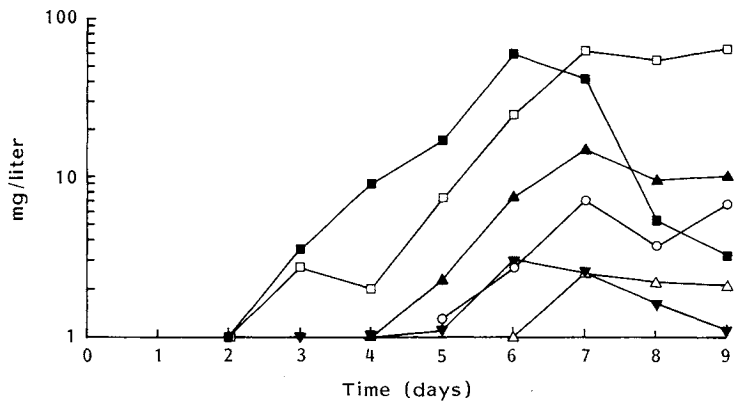


Experimental conditions: Analytical silica column (Lichrosorb Si60, 5 μm; 4×250 mm), hexane - *tert*-butylmethyl ether (80:20; 2 ml/minute) monitored at 300 nm; 14 μg in 20 μl ethyl acetate injected.

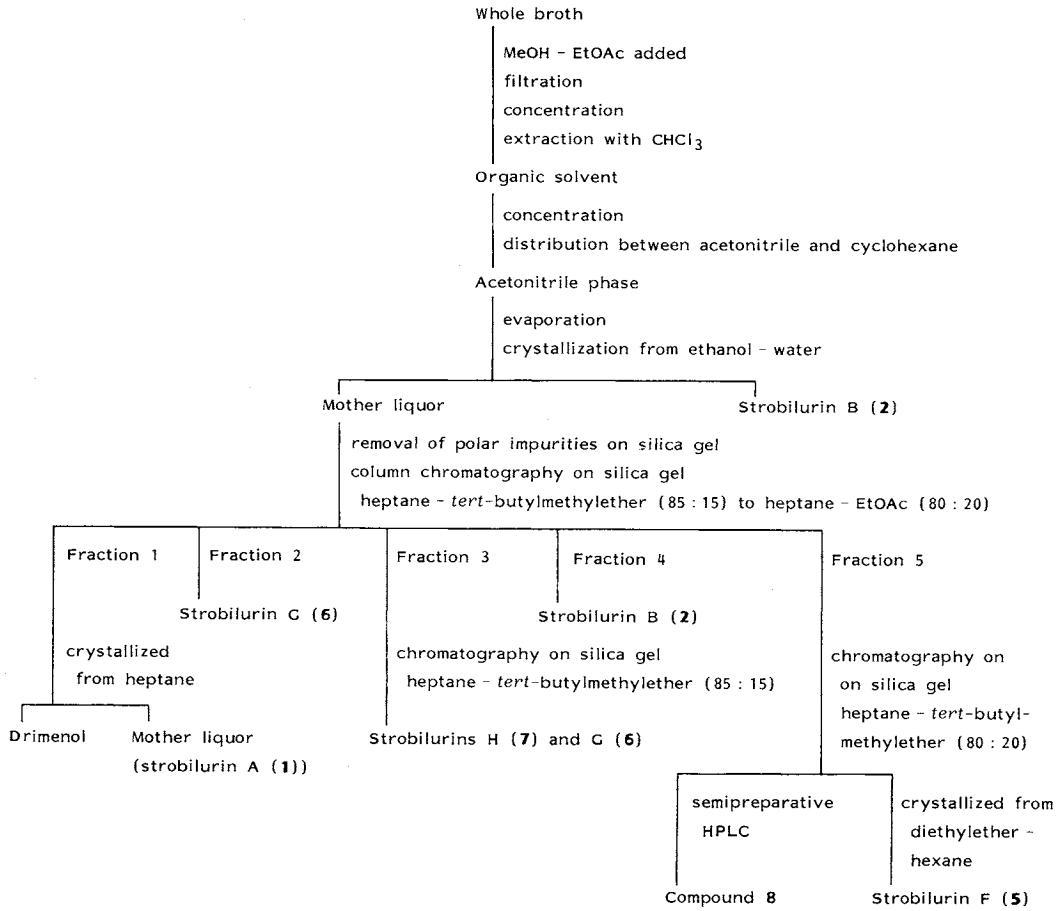
Results and Discussion

In the fermentation broth of *B. lutea* Sacc. the strobilurins are mainly found in the mycelium. Extraction of the whole broth and partitioning between acetonitrile and cyclohexane gave a material which was sufficiently pure to allow the separation of the main metabolite strobilurin B by crystallization. The new strobilurins and compound **8** were

Fig. 2. Time couse of the production of the compounds **1**, **2** and **5** to **8** in shake flasks.
■ **1**, □ **2**, △ **5**, ○ **6**, ▲ **7**, ▼ **8**.



Scheme 2. Isolation procedure for strobilurins A, B, F, G, H and compound **8**.



obtained by repetitive chromatography of the mother liquor on silica (Scheme 2). Isolation and analysis of shake flasks experiments were assisted by an analytical HPLC method (Fig. 1). The structure elucidation and the physico-chemical data of these compounds will be described in the subsequent paper[14].

Shake flask experiments and analysis of the time course of the product formation by HPLC (Fig. 2) lead to interesting insights into the biosynthesis of the strobilurins: Compound **8** seems to be produced at a similar stage of the fermentation as strobilurin A. Therefore **8** cannot be considered as a degradation product but rather as an unwanted side-product arising during the biosynthesis of strobilurin A or as a precursor for the latter. Strobilurin A contains one additional carbon atom. According to labeling studies on the biosynthesis of strobilurin A by NERUD *et al.* [1-^{13}C]acetate is incorporated into the missing C-13 as well as into the adjacent C-11[15]. It can be theorized that C-13 might be derived from an intramolecular migration of a terminal carboxyl function as demonstrated for tropic acid[16]. In that case compound **8** would be formed by decarboxylation of a hypothetical intermediate.

Strobilurin A is not stable in a culture liquid and seems to be converted to the other strobilurins (**2** and **5**~**7**), which are all produced at approximately the same time. Strobilurin H (**7**) lacks one chlorine atom of **2** and might be a biosynthetic intermediate between strobilurins A and B, suggesting that **2** is halogenated at a very late stage. Strobilurins F and G are formed from **1** by addition of one or two

Table 1. Antimicrobial spectra of the strobilurins B, F, G and H.

Test organism	MIC (μg/ml)[a]			
	B (**2**)	F (**5**)	G (**6**)	H (**7**)
Staphylococcus aureus 10B	>128	>128	>128	>128
Candida albicans ATCC 11651	>128	>128	>128	>128
C. tropicalis ATCC 13803	>128	>128	>128	>128
Torulopsis glabrata H 556	>128	>128	>128	>128
Blastomyces dermatitidis ATCC 26199	2	2	1	
Trichophyton mentagrophytes ATCC 9533	4	16	128	32
T. quinckeanum D 24	2	4	8	64
Microsporum canis ATCC 10214	4	32	32	64
Aspergillus fumigatus ATCC 9197	>128	>128	>128	>128
Sporotrichum schenckii ATCC 10212	>128	>128	>128	128
Botrytis cinerea[b]		20	<20	40
Cercospora arachidicola ATCC 18667[c]		>200	100	100

Test organism	Inhibition zone (mm) with 1/0.1 μg antibiotic per disc			
	B (**2**)	F (**5**)	G (**6**)	H (**7**)
Cephalosporium acremonium C 1100	30/21	20/11	11/0	34/19
Paecilomyces variotii Tü 137	0/0	0/0	0/0	0/0
Piricularia oryzae AC 164	13/13*	0/0	13/10	24*/12*
Pythium debaryanum CBS 26538	11*/0	9/0	0/0	12*/0
Rhizoctonia solani ATCC 18184	15*/0	0/0	0/0	0/0

[a] Agar dilution assay.
[b] Tested on apples.
[c] Tested on peanut plants.
* Indicates inhibition zones which are not completely clear.

Table 2. *In vitro* antitumor activity of the strobilurins F (**5**), G, (**6**), H (**7**) and **8**[a].

	5	**6**	**7**	**8**
Antiproliferative activity IC_{50} (μM)	2.2	0.01	2.5	79

[a] Cellular pharmacology on T-24 human bladder carcinoma.

isoprene units, respectively.

Biological Properties

The antimicrobial spectra of the strobilurins is shown in Table 1. They are strong fungicides but less active against yeasts. The four tested strobilurins exhibit marked differences against the tested strains. Generally strobilurin B is the most active fungicide. *In vitro* antitumor activity (Table 2) of strobilurin G (**6**) against the T-24 human bladder carcinoma cell line[17] has by two orders of magnitude a greater activity than the other compounds tested.

Acknowledgments

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