# 8006-I, AN ANTIBIOTIC FROM *AMBLYOSPORIUM SPONGIOSUM* (PERS.) HUGHES SENSU PIROZYNSKI

## I. TAXONOMY, FERMENTATION, ISOLATION AND PHYSICO-CHEMICAL PROPERTIES*

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A strain of *Amblyosporium spongiosum* (PERS.) Hughes sensu Pirozynski, HA 8006 isolated from an infected *Lactarius deliciosus* was found to produce two antibiotics, 8006-I and 8006-II. The same compounds could be isolated from cultures of several strains of the genus *Amblyosporium* obtained from the Centraalbureau voor Schimmelcultures.

The highest yields of 8006-I (80 mg/liter culture) were obtained from the mycelium of strain HA 8006. Antibiotic 8006-I is unstable in the presence of light or oxygen and has a carotenoid structure.

During our screening of saprophytic or parasitic fungi growing on basidiomycetes the strain HA 8006, *Amblyosporium* sp., was found to produce two antibiotics, 8006-I and 8006-II. To our knowledge strains of the genus *Amblyosporium* have not been reported to produce secondary metabolites. This paper deals with the taxonomy of the producing strain, the fermentation, isolation, and physico-chemical properties of 8006-I. The biological properties of 8006-I will be described in the subsequent paper, and 8006-II will be described in a separate paper.

### Materials and Methods

#### Cultivation of Test Organisms

*Bacillus brevis* and *Proteus vulgaris* were grown and tested on nutrient broth (Difco); *B. subtilis* was grown on nutrient broth (NB) and tested on a synthetic medium (MM2) composed of (per liter): $KH_2PO_4$ 3.0 g, $K_2HPO_4$ 7.0 g, $(NH_4)_2SO_4$ 1.0 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, NaCl 0.1 g and glucose 2.0 g (sterilized separately). The pH was adjusted to 7.2 prior to sterilization. For solid media Bacto-agar (Difco), 20 g, were added to one liter of medium.

#### Fermentation of *Amblyosporium spongiosum* HA 8006

The strain was kept on YMG medium (yeast extract 4 g, malt extract 10 g, glucose 4 g, agar 20 g, per liter). The production medium (PM) was composed of (per liter): Asparagine·$H_2O$ 10 g, glucose 50 g, $KH_2PO_4$ 5 g, $MgSO_4 \cdot 7H_2O$ 5 g, HOAGLAND mineral salt solution[2] 1 ml. The pH was adjusted to 3.8 prior to sterilization.

Erlenmeyer flasks with one intrusion containing 150 ml of PM medium were inoculated with freshly prepared spores ($10^6$ spores/flask) and incubated at 27°C on a rotary shaker (120 rpm) for 10~14 days.

Fermentors were inoculated with 15% of a well grown mycelial culture. Fermentations were carried out in a type b 20 vessel (Braun Melsungen AG, Melsungen, FRG) with "intensor system", 800 rpm;

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5 liters air/minute; 27°C. To prevent foaming, Niax polypropylenglycol (Union Carbide) was added. During fermentations the pH was measured with a glass electrode (Inogold, Zürich, Switzerland). The dry weight of the mycelium was determined as described by KAPPNER *et al.*[3]. For the determination of glucose in the culture broth, Glucotest from Boehringer and Söhne, Mannheim, was used.

The following strains were purchased from the Centraalbureau voor Schimmelcultures, Baarn (CBS) *Amblyosporium botrytis* (FRES.) strain 115.35 and strain 331.67; *Amblyosporium spongiosum* (PERS.) Hughes sensu Pirozynski strain 107.08, strain 548.69, and strain 502.74. According to its morphological features *Amblyosporium botrytis* strain 115.35 should be transferred to *Amblyosporium spongiosum* (PERS.) Hughes sensu Pirozynski. This strain has been deposited prior to the reassessment of the genus *Amblyosporium* by PIROZYNSKI[4].

General Methods

IR spectra were determined on a Perkin-Elmer 21 spectrometer and UV spectra on a Beckmann DB-GT. Mass spectra were recorded on a Varian MAT 731 (70 eV) using perfluorokerosine as reference for high resolution measurements.

## Results and Discussion

### Taxonomic Features of the Producing Strain

The strain HA 8006 was isolated from an infected *Lactarius deliciosus* (L. *ex* FR.) S. F. Gray collected near Tübingen. It grows readily on most common agar media like malt extract (MA), oatmeal (OM), potato dextrose (PD), corn meal (CM), NB, or YMG at temperatures ranging from 18°C to 30°C. Growth is best at 27°C and no growth was observed on CZAPEK-DOX (CD) or RAULIN-THOM (RT) agar at 37°C. On all media the mycelium is brightly orange colored and the strain sporulates abundantly. According to the morphological features of the conidiophores and the conidiospores it was determined to belong to the form genus *Amblyosporium*[5,6], species *spongiosum*[4]. In addition to the orange colored strain HA 8006, a white variant of *Amblyosporium spongiosum* was isolated from the infected mushroom. In this white strain no production of antimicrobial metabolites was observed.

Several strains of the genus *Amblyosporium* obtained from CBS, were also found to produce antibiotics. In Tables 1 and 2 the ability to grow, to sporulate and to produce antibiotics of our strain is compared with those of the strains obtained from CBS. Since most of the strains have been deposited

Table 1. Comparison of growth and sporulation of six different strains of the genus *Amblyosporium* on solid media at 27°C.

| Medium | CBS 331.67 | | CBS 548.69 | | CBS 107.08 | | CBS 115.35 | | CBS 502.74 | | HA 8006 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | G* | S | G | S | G | S | G | S | G | S | G | S |
| YMG | ++ | + | ++ | − | + | − | ++ | ++ | ++ | ++ | ++ | ++ |
| MA | + | − | ++ | − | + | − | ++ | + | ++ | ++ | ++ | ++ |
| RT | − | | − | | − | | − | | − | | − | |
| CD | − | | − | | − | | − | | − | | − | |
| OM | + | − | + | − | − | − | + | − | + | + | ++ | ++ |
| PD | − | | − | | − | | + | − | ++ | + | + | + |
| CM | + | − | + | − | + | − | + | + | + | + | + | ++ |

* G: growth
− no growth
\+ thin layer of mycelium
++ thick layer of mycelium

S: sporulation
− no conidiophores detectable
\+ <10 conidiophores/cm²
++ >20 conidiophores/cm²

Table 2. Comparison of growth and production of antibiotics of six different strains of the genus *Amblyosporium* in submerged culture at 27°C.

| Strain | PM medium growth | YMG medium growth | Color of mycelia | Antibiotic production on YMG | | | |
|---|---|---|---|---|---|---|---|
| | | | | 8006-I (mg/liter) | | 8006-II (mg/liter) | |
| | | | | Filtrate | Mycelium | Filtrate | Mycelium |
| HA 8006 | ++ | +++ | Rust | 1.8 | 22.2 | 1.0 | 1.5 |
| 502.74 | − | +++ | Rust-brown | 0.8 | 2.7 | 0.0 | 0.6 |
| 115.35 | − | +++ | Rust-brown | 1.2 | 6.5 | 0.6 | 1.0 |
| 331.67 | − | +++ | Rust-brown | 0.6 | 6.9 | 0.5 | 0.5 |
| 107.08 | − | ++ | Grey-brown | 0.4 | 2.7 | 0.2 | 0.3 |
| 548.69 | − | +++ | Pale rust | 1.5 | 5.0 | 0.7 | 0.8 |

at CBS prior to the reassessment of the genus *Amblyosporium* by PIROZYNSKI[4], we have listed the strain number only in order to avoid confusion. The names under which the strains have been deposited are given in Materials and Methods. For comparison the antibiotics were extracted from the culture filtrates with ethyl acetate and from the mycelia with acetone. The antibiotic content of the extracts was determined by TLC and by bioautography using *Bacillus subtilis* (MM2), *Proteus vulgaris* and *B. brevis* (NB) as test organisms, and by recording the absorption at 452 nm (8006-I). As shown in Tables 1 and 2, our strain HA 8006 sporulates much better and produces higher amounts of antibiotics as compared to the strains obtained from CBS. *Amblyosporium spongiosum*, CBS 502.74 (deposited in 1974), also sporulates abundantly, however, the yield of antibiotics from this strain is lower. Therefore all further work was carried out with our strain HA 8006. The search for a medium yielding higher amounts of 8006-I led to a synthetic medium (PM) with asparagine and glucose as carbon- and nitrogen source. As shown in Table 2, strain HA 8006 was the only one to grow in this medium.

## Production and Isolation of 8006-I

A typical fermentation diagram of *Amblyosporium spongiosum* HA 8006 in a 25-liter fermentor is shown in Fig. 1. The production of 8006-I was observed after two or three days of cultivation and reached its maximum after 8 ~ 10 days, thereafter the antibiotic content of the mycelium decreased rapidly, and some antibiotic activity could be detected in the culture filtrate due to hyphal breakdown. At the

Fig. 1. Fermentation diagram of *Amblyosporium spongiosum* HA 8006 grown on PM medium.

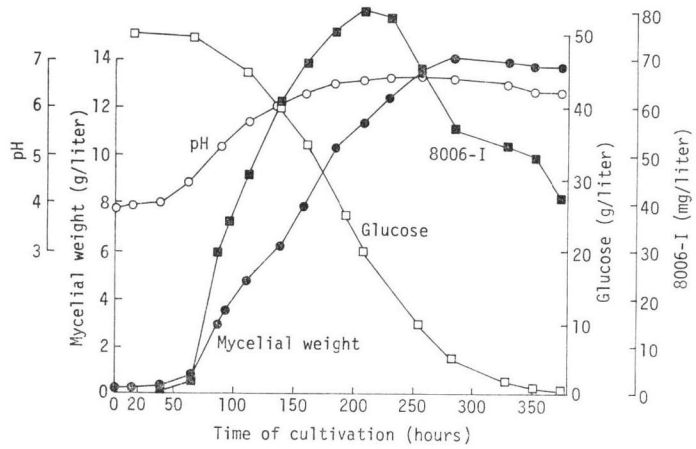
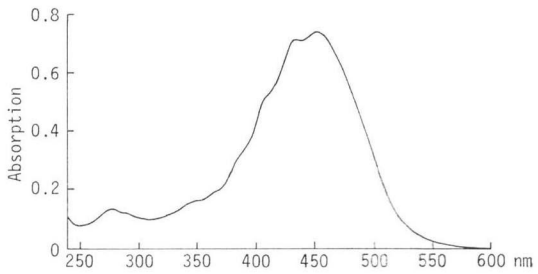


Table 3. Minimum inhibitory concentrations (MIC) of 8006-I against the test organisms.

| Organism | Medium | MIC ($\mu$g/ml) |
|---|---|---|
| *Bacillus brevis* ATCC 9999 | NB | 5~10 |
| *Bacillus subtilis* ATCC 6051 | NB | 10 |
| 〃 〃 | MM2 | 5 |
| *Proteus vulgaris* | NB | 10 |

Serial dilution assay; $10^6$ cells/ml; 37°C.

Fig. 2. UV absorption spectrum of 8006-I in chloroform.




same time, the formation of an insoluble orange colored precipitate was observed. The cultures were harvested when the pH value had reached its plateau (pH 6.3). The mycelia were separated using a Büchner funnel and extracted three to four times with acetone. The culture filtrate containing less than 10% of the total activity was discarded. The mycelial extracts were concentrated and the aqueous residue was extracted with petroleum ether (60~80°C). The pale yellow organic phase was discarded and the orange colored antibiotic was extracted from the aqueous solution with chloroform. The chloroform extracts were concentrated and applied on to a silica gel column. The column was kept in the dark and the antibiotic was eluted with chloroform. Final purification was achieved by preparative LC (Merck silica plates, Nr. 5745) in chloroform - methanol (9: 1) in the dark. The antibiotic content of the extracts was measured in the agar diffusion assay using *P. vulgaris, B. brevis* or *B. subtilis* as test organisms or by recording the absorption at 452 nm ($\Delta E^{1\%}_{1cm}$ MeOH: 1580).

The minimum inhibitory concentrations for the test organisms are given in Table 3.

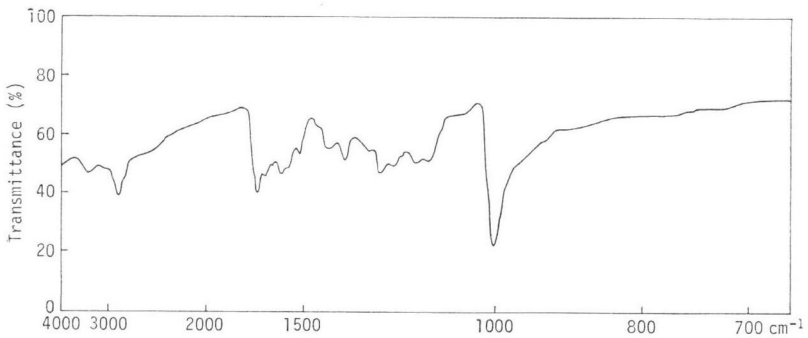
The antibiotic was stored as a solution (2 mg/ml) in MeOH or acetone at −20°C.

## Physico-chemical Characterization

Antibiotic 8006-I can be crystallized from chloroform - methanol yielding rust colored prisms. Melting point: 125°C (browned), 160°C (decomposition). It is soluble in acetone, methanol, and ethanol; less soluble in chloroform and almost insoluble in water. The UV spectrum (chloroform) is given in Fig. 2 [$\lambda_{max}$ ($E^{1\%}_{1cm}$): 452 (2200), 433 (2100), 408 nm (sh 1550)]. In EtOH the UV spectrum shows a broad maximum at 435 nm; upon addition of acid (HCl) or base (NaOH) no shift of the spectrum was observed.

The infrared spectrum is depicted in Fig. 3.

Fig. 3. Infrared absorption spectrum of 8006-I (KBr).



The deep orange coloration of a solution of 8006-I in acetone or other organic solvents disappeared upon standing at room temperature in daylight. After one day orange precipitates were formed and after 8~10 days the solutions became colorless. Concurrently to the loss of color the antibacterial activity disappears. At −20°C in the dark the solutions could be kept for 4~6 weeks without loss of activity, although later on the formation of biologically-inactive precipitates were also observed.

Table 4. Chromatographic behaviour of 8006-I.

| Solvent system | Rf |
|---|---|
| $CHCl_3$ | 0.04 |
| $CHCl_3$ - MeOH, 97: 3 | 0.19 |
| $CHCl_3$ - MeOH, 90: 10 | 0.65 |
| *n*-BuOH - EtOH - $H_2O$, 4: 1: 5 | 0.69 |

Silica plates (Merck 5554)

The Rf values on silica gel TLC in different solvents are given in Table 4. Besides its orange color, 8006-I can be recognized by its reaction with a saturated solution of $SbCl_3$ in chloroform (Carr-Price reagent[7]) — the orange spots turn dark blue, the reaction can be intensified by heating — and by the transient blue coloration after spraying with concentrated sulfuric acid or trichloroacetic acid. All reactions indicate a carotenoid structure or structure component[8].

The elementary analysis yielded 74.48% C and 7.13% H, no nitrogen or halogens.

No mass spectrum could be obtained from the antibiotic 8006-I. Methylation with diazomethane (in ether) yielded a methyl ester and from the trimethylsilyl ether derivative of this ester a mass spectrum could be obtained. $C_{26}H_{34}O_3Si$ was calculated for the trimethylsilyl ether derivative and $C_{23}H_{26}O_3$ for the methyl ester of 8006-I. Further experiments were necessary to confirm the molecular formula of $C_{22}H_{24}O_3$ for 8006-I. None of the known fungal carotenoids[9,10] has a molecular formula identical to 8006-I, and strains of the genus *Amblyosporium* have not been investigated for the production of carotenoids.

The elucidation of the structure of 8006-I is currently under investigation.

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