Epothilons A and B: Antifungal and Cytotoxic Compounds from *Sorangium cellulosum* (Myxobacteria)

Production, Physico-chemical and Biological Properties†

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An antifungal activity against *Mucor hiemalis* was detected in the culture broth of *Sorangium cellulosum* (Myxococcales) strain So ce90. The activity was excreted into the supernatant during the log and early stationary phase. When the adsorber resin XAD-16 was added to the culture, the active metabolites were quantitatively bound to the resin. The epothilons showed a high cytotoxicity for animal cells and mimic the biological effects of taxol (BOLLAG *et al.*, Cancer Res. 55: 2325~2333, 1995).

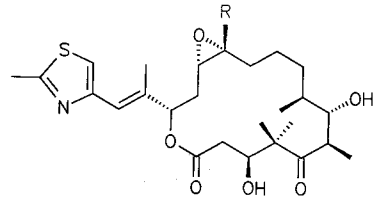
In our screening program for secondary metabolites from myxobacteria, we detected metabolites with a narrow antifungal spectrum: they were inhibitory for *Mucor hiemalis* only. They were first seen in the culture broth of *Sorangium cellulosum* strain So ce90 and turned out to be novel macrocyclic polyketides[1].

This paper deals with the production, identification and biological characterization of the new activity from strain So ce90. The structure elucidation of epothilons A and B (Fig. 1) is reported elsewhere[2].

Microorganism and Culture Conditions

The producing organism, *Sorangium cellulosum* So ce90, was isolated at the GBF in 1985 from a soil sample collected at the banks of the Zambesi river in the Republic of South Africa. Stock cultures were kept on yeast agar plates (VY/2-agar: bakers' yeast, 0.5% by fresh weight of yeast cake; $CaCl_2 \cdot 2H_2O$, 0.1%; vitamin B_{12}, 0.5 mg/liter; agar, 1.5%; pH 7.2; autoclaved). The strain formed large swarm colonies with many yellowish-orange to black-brown fruiting bodies. The fruiting bodies consisted of small sporangioles, 15~20 μm in diameter, which were tightly packed in more or less large masses, or sori. The sori were usually between 50 and 150 μm in diameter. The vegetative rods were of the Sorangineae type: fairly compact dark, cylindrical rods with broadly rounded ends, on average 3~6 μm long and 1 μm thick. The strain grew in homogeneous cell suspension after a relatively long adaption phase. Liquid cultures were started by inoculating the bacterium into 250-ml Erlenmeyer flasks containing 100 ml of the following production medium (in g/liter distilled water): potato starch (Maizena), 8; glucose (Maizena), 2; defatted soybean meal, 2; yeast extract (Marcor), 2; ethylendiamine-tetraacetic acid, iron(III)-sodium salt, 0.008; $MgSO_4 \cdot 7H_2O$, 1; $CaCl_2 \cdot 2H_2O$, 1; HEPES 11.5. The pH of the medium was adjusted to 7.4 with KOH before autoclaving. For continuous adsorption of lipophilic metabolites, 2% (v/v) of XAD-16 (Rohm and Haas, Frankfurt/M) was added. So ce90 grew in homogeneous cell suspension to a high cell density, up to $2 \cdot 10^9$ cells/ml, with a generation time of 16 hours during the log phase.

Fig. 1. The structures of epothilon A and B.



Epothilon A R=H
B R=CH_3

† Art. No. 74 on antibiotics from gliding bacteria. Art. No. 73: BÖHLENDORF, B.; E. FORCHE, N. BEDORF, K. GERTH, H. IRSCHIK, R. JANSEN, B. KUNZE, W. TROWITZSCH-KIENAST, H. REICHENBACH & G. HÖFLE: Liebigs Ann. Chem. 1996: 49~53.

Production

A 100-liter bioreactor (Giovanola Frères, Monthey, Switzerland) with 60 liter of the production medium (as above, but without HEPES) was inoculated with 5 liter of a 4-days old preculture grown under shaking (160 rpm, 30°C) in the same medium in 1-liter Erlenmeyer flasks containing 500 ml medium. To prevent foam formation, 10 ml silicone antifoam (Tegosipon, Goldschmidt AG, Essen) was added. The fermentation was run for 3 days at 32°C, with an aeration rate of 300 liters of air per hour and a stirrer speed of 250 rpm. The pH was maintained at 7.4 with 10% KOH.

This seed fermentor was used to inoculate a 350 liter fermentor containing 230 liter of production medium (without HEPES) and 2% of adsorber resin XAD-16. The fermentation was run for 7 days at 32°C and pH 7.4, with an aeration rate of 1 m^3 of air per hour and a stirrer speed of 350 rpm. The epothilons were produced during the log phase up to the stationary phase of growth. At the end of the fermentation, 22 mg/liter of epothilon A and 11 mg/liter of epothilon B were determined by HPLC analysis.

The adsorber resin was separated from broth and cells with a process filter. After washing the resin with water, the active material was eluted with four bed volumes of methanol. The extract was then concentrated *in vacuo* at 40°C. From the remaining water phase, the active material was reextracted with ethyl acetate. The isolation of the epothilons by chromatography and crystallization is described in detail elsewhere[2].

Physico-chemical Properties

The antibiotic was analysed by thin-layer chromatography (Silica gel Si 60 F_{254}, Merck, Darmstadt) with dichloromethane - methanol (90 : 10) as the solvent. The epothilons A and B did not separate in this system and were detected at an Rf value of 0.75 by their UV absorption. For HPLC analysis, a Lichrosorb RP-18, 7 μm column, 4 × 250 mm (Fa. Merck, Darmstadt), was used. The solvent was methanol - water (65 : 35), 1.5 ml/minute. Epothilon A was detected after 5.4 and epothilon B after 6.3 minutes by absorption at 254 nm.

Fig. 2 shows the electronic absorption spectrum of epothilon A in methanol . Crystals of pure epothilon A and B had melting points of 95°C and 93~94°C, respectively.

The IR spectrum of epothilon A in KBr (Fig. 3) was measured with an FT-IR spectrometer 20 DXB (Nicolet), the ^1H NMR spectrum in $CDCl_3$ (Fig. 4) with an AM-400

Fig. 2. Electronic absorption spectrum of epothilon A in methanol.

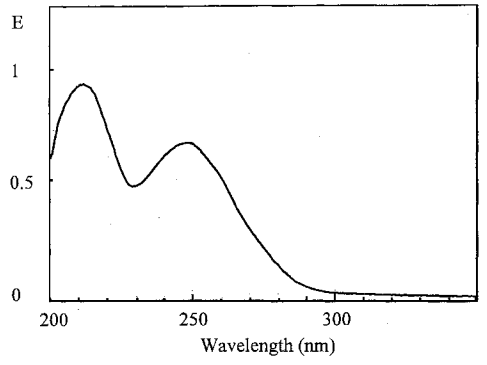


Fig. 3. IR spectrum of epothilon A in KBr.

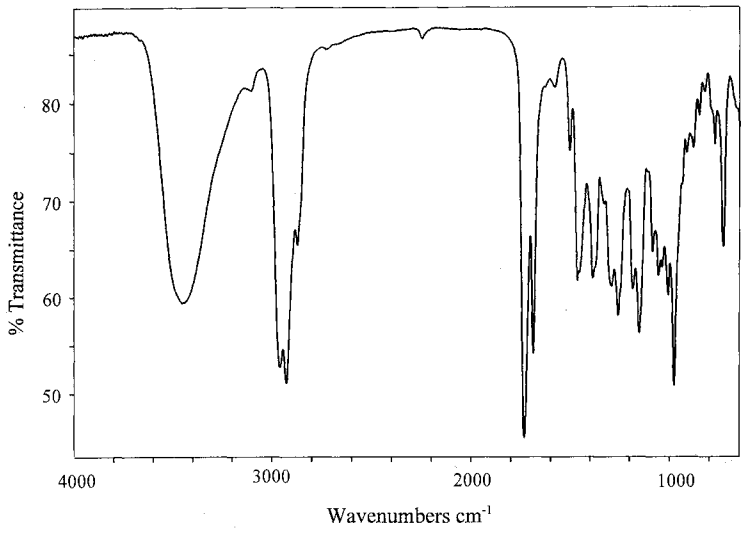
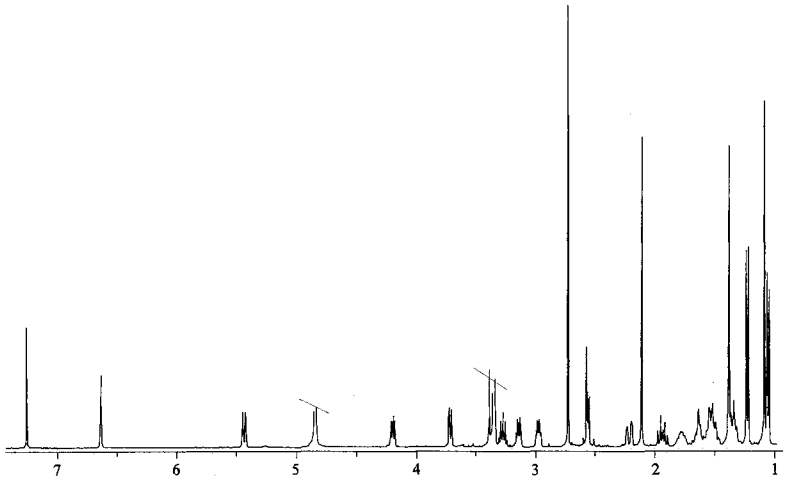


Fig. 4. ^1H NMR spectrum of epothilon A in $CDCl_3$.



400 MHz ^1H NMR spectrum of epothilon A in CD_3OD (Bruker WM-400 spectrometer)

spectrometer 400 MHz (Bruker, Karlsruhe). The FAB-MS (negative ions) gave 492.25 $(M-H)^-$ and 506.25 for epothilons A and B, respectively, which is in agreement with the elemental compositions of $C_{26}H_{39}NO_6S$ and $C_{27}H_{41}NO_6S$ derived from spectroscopic data and elemental analysis[2].

Biological Properties

The antimicrobial spectrum was determined by the paper disk method. Bacteria were not inhibited. Among the numerous yeasts and fungi which were tested *in vitro*, only the zygomycete, *Mucor hiemalis*, was sensitive to the inhibitors. However, in greenhouse experiments, important plant pathogenic fungi were inhibited[3], especially various oomycetes, like *Pythium ultimum*, *Plasmopara viticola* and *Phytophthora infestans*[4]. The minimum inhibitory concentrations (MICs) against *M. hiemalis*, determined by the serial dilution assay in liquid culture, were 20 μg/ml for both components. The IC_{50} for mouse fibroblasts (line L929) were 15 ng/ml for epothilon A and 2 ng/ml for epothilon B. The IC_{50} for the human T-24 bladder carcinoma cell line was 0.05 μM for epothilon A[3].

Discussion

Our screening of 700 *Sorangium cellulosum* strains revealed that 1.6% of the isolates synthesized epothilons, as identified by their biological effects and by HPLC. Among those strains, 79% were at the same time producers of spirangiens[5], and 21% excreted icumazols[6]. Like ratjadon[7], the epothilons have a narrow antifungal spectrum but show a high cytotoxicity in animal cell cultures. Such a high toxicity combined with high selectivity indicates a very specific interference with essential steps of cellular development. While ratjadon, like structurally related leptomycin[8], may interfere with the maintenance of chromosome structure, the epothilons appear to mimic rather precisely the effects of taxol, *i.e.*, they stabilize the microtubules by binding to them[9]. An important difference between the two compounds is that, in contrast to taxol, epothilon is considerably less efficiently exported from the cells by P-glycoprotein. Epothilon and taxol also match in their selective action on oomycetes[10]. The studies on the mechanism of action just mentioned were performed with epothilon discovered independently, but after publication of our patent[3], in a screening of 7000 extracts specifically for substances that mimic the taxol effects[11]. In this case, too, the producer was a strain of *So. cellulosum*. It may be expected that the epothilons, which can be obtained relatively easily by fermentation, will also become useful as antitumor agents in the future.

Acknowledgments

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