PRODUCTION OF CLADOSPIRONE BISEPOXIDE, A NEW FUNGAL METABOLITE

FRANK PETERSEN*, THÉOPHILE MOERKER, FORTUNATO VANZANELLA[†] and HEINRICH H. PETER

Biotechnology Research Laboratories, Pharmaceutical Division, CIBA-GEIGY Limited, CH-4002 Basel, Switzerland
[†]Division of Fermentation Products, CIBA-GEIGY Limited, Torre Annunziata (NA), Italy

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Cladospirone bisepoxide (**1**), a novel metabolite, was isolated from cultures of a fungus which was characterized as a coelomycete by the formation of pycnidia. By optimization of media and fermentation conditions, a titer of up to 1.5 g/liter on shake level and 1.16 g/liter on bioreactor scale could be achieved. The isolation of the compound was performed by solvent extraction of the culture broth and subsequent crystallization. Cladospirone bisepoxide displays selective antibiotic activity against several bacteria and fungi and inhibits germination of *Lepidium sativum* at low concentrations.

In the course of our screening program for new secondary metabolites, we investigated culture extracts of the fungus strain F-24'707, initially classified as a *Cladosporium*. A recent revision[††], however, showed that the strain could be induced to form pycnidia in culture and should be classified as a coelomycete. This strain produced a unique active substance which was named cladospirone bisepoxide, since it contained a spirocyclic bond to a 1,8-dihydroxy-naphthalene residue and two epoxide groups. The proposed structure of this metabolite was based on spectroscopic data and the X-ray structure analysis of its keto-derivative (**2**)[1], Fig. 1. In this paper, we describe the fermentation of the producing microorganism, the isolation procedure and some of the biological activities of cladospirone bisepoxide (**1**).

Materials and Methods

Strain Isolation, Morphology, and Maintenance

The saprophytic fungus strain F-24'707 was isolated from a bed of needles collected in a forest of Montezuma pines about 200 km north of Mexico City at an altitude of 3,000 m above sea level. On oatmeal agar, numerous pycnidia with a thin wall were formed. Conidia were built from simple, bottle-shaped phialides in the inner wall of the pycnidia. The conidia were cylindric, $4.5 \times 2\,\mu m$ big and, in masses, creamish. Clamydospores were not observed. Cultures of this strain were grown on LCSB agar with following ingredients: Lactose 1.5%, cornsteep liquor 0.5%, peptone 0.5%, NaCl 0.4%,

Fig. 1. Tentative chemical structure of cladospirone bisepoxide (**1**) and structural formula of the diketone (**2**) as confirmed by X-ray structure analysis.

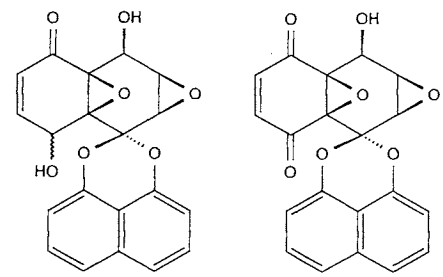

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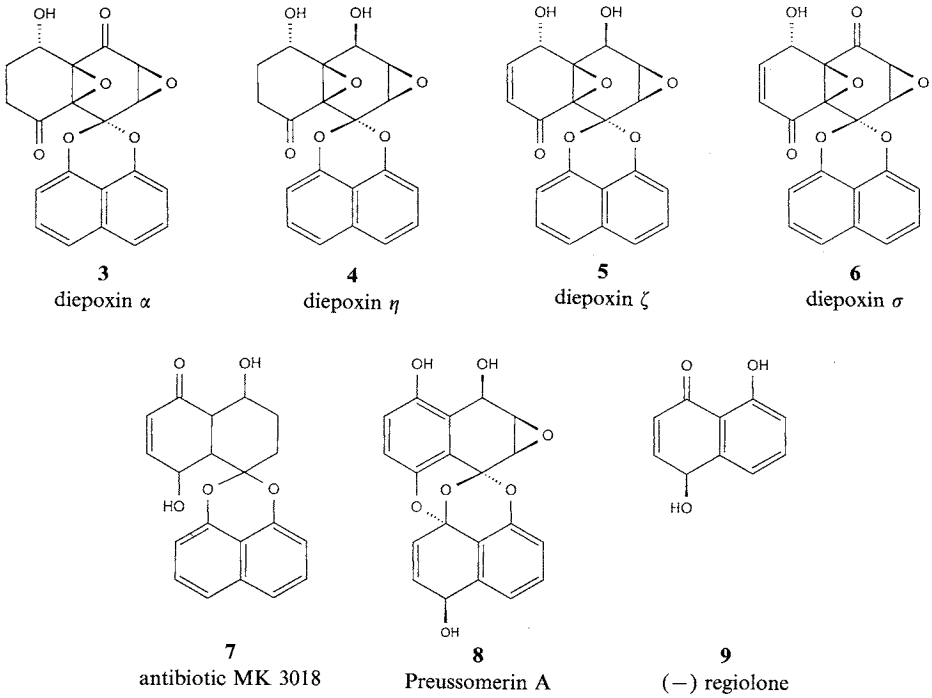
Fig. 2. Chemical structures of related fungal metabolites.



$MgSO_4 \times 7H_2O$ 0.05%, KH_2PO_4 0.06%, $FeCl_3 \times 6H_2O$ 0.0005%, $CuSO_4 \times 5H_2O$ 0.0002%, and agar 3.0%. Before sterilization, the pH was adjusted to 4.8 with sulfuric acid (98%) or sodium hydroxide (30%). The slants were incubated at 28°C for 10 days until complete sporulation had occurred. Working stocks are stored at 4°C whereas kryo ampoules are kept at −80°C and −169°C respectively.

Fermentation

A mature slant culture of the strain F-24'707 was inoculated into a 500-ml Erlenmeyer flask with 1 baffle containing 100 ml of a medium with the following composition: Pharmamedia (Archer Daniels Midland Co., Memphis, U.S.A.) 0.3%, NH_4NO_3 1.0%, soybean meal (defatted) 0.5%, fish protein 2.0%, yeast extract (Difco, Detroit, U.S.A.) 0.3%, proteose peptone (Difco) 0.3%, glucose 4.0%, dextrin 2.0%, glycerol 2.0%, malt extract (Difco) 1.0%, KH_2PO_4 0.06%, $CaCO_3$ 1.0%. The pH was not adjusted. The culture was shaken under aerobic conditions for 96 hours at 28°C and 250 rpm. 5 ml of the seed culture was transferred into 500 ml of the above medium in a 2,000-ml Erlenmeyer flask with four baffles. The vegetative culture was incubated under the same conditions as already described. 5.0% of the second seed culture was transferred into a 3.5 liters fermenter (MBR), driven by blade stirrer, containing 1.5 liters of the following production medium M-4: Sucrose 2.0%, soybean meal (defatted) 2.0%, oat meal (Klingentalmühle AG, Kaiseraugst, Switzerland) 2.0%. The pH was not adjusted before sterilization. The fermentation was carried out at 28°C for 48 hours with a aeration rate of 1.5 liters/minute and an agitation rate of 700 rpm. At harvest time, the PCV (packed cell volume) of the culture reached 46% and the titer a value of 1.16 g/liter as determined by HPLC analysis.

Analytical Chromatography and UV Spectrum Determination

The concentration of cladospirone bisepoxide was determined using a reversed-phase HPLC method. The following equipment was used: Autosampler (Jasco, mod.: 851-AS), low pressure gradient forming HPLC pump (Merck, mod.: L 6200 A), photometric detector (Shimadzu, mod.: SPD-6 AV, wavelength set to 220 nm). The separations were performed using Lichrospher RP-18 as stationary phase in a steel column (4.0 × 100 mm). The mobile phase A was 2.5 mM K-phosphate buffer adjusted to pH 3.0 and phase

B consisted of 80% acetonitrile mixed with 20% of phase A. The analysis was carried out with a flow rate of 1.5 ml/minute, an injection volume of 25 μl, and a gradient run from 5 to 100% phase B within 25 minutes. For the preparation of test vials, 5 ml of the culture broth was mixed with an equal volume of 2-propanol, stirred for 30 minutes, and centrifuged. The clear supernatant was injected for HPLC analysis.

The UV spectrum of cladospirone bisepoxide were determined using a UV photometer (Perkin-Elmer, mod.: Lambda 5). The measurement was carried out with an ethanolic solution of 11.5 μM cladospirone bisepoxide.

Isolation Procedure

The highly viscous dark colored culture broth (1.3 liters) from the above described fermentation on bioreactor scale was transferred to a flask containing 3 liters of a solvent mixture of ethyl acetate - methanol in the ratio of 9 : 1 and stirred at room temperature during 1 hour. Since no separation of the phases could be observed 500 g of filter and (Hyflo) was added. The cell material was removed by filtration and washed with a total of 1 liter of ethyl acetate. After saturation with sodium chloride a clear separation of the phases occurred. The aqueous phase was separated and reextracted with 1.5 liters of the ethyl acetate - methanol (9 : 1) mixture. The two extracts were separately dried over anhydrous sodium sulfate and evaporated to dryness. The desired product was almost completely concentrated in the first extract. The second extract (0.1 g) containing a complex mixture of components was discarded.

Recrystallization of the first extract from 20 ml of methanol gave 1.247 g of chromatographically pure product. The mother liquors were concentrated and triturated with 50 ml of *n*-heptane. The insoluble residue was crystallized from 10 ml of methanol yielding 0.053 g of additional material. The total amount of crystals (1.300 g, isolation yield: 86%) was recrystallized and dried in the high vacuum. The slightly greyish needles (mp 160 dec.) were characterized by HPLC and spectroscopic methods and found indistinguishable from the material, which had been purified by column chromatography on silica gel as described in a previous publication[1]. The UV-spectrum is given in Fig. 3.

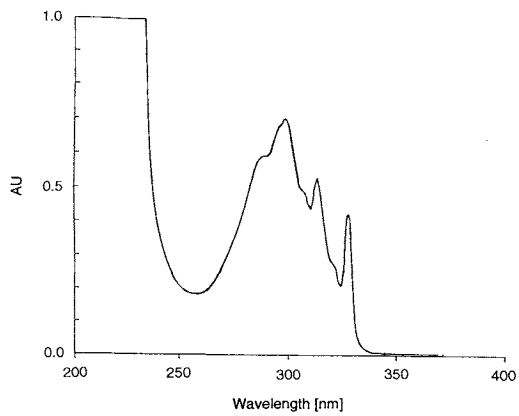
Antibiotic Activity

The test organisms listed in Table 2 were cultivated in one the following media: Medium G-13: Oat meal 2.0%, yeast extract (Difco) 0.4%, malt extract (Difco) 1.0%, glucose 0.4%, pH 7.3 (adjusted with 5N NaOH); Brain Heart Infusion Agar (Difco); D.S.T. Agar (Oxoid); Mycophil Agar (BBL); Bss-agar; trisodium citrate 0.05%, potassium dihydrogen phosphate 0.35%, disodium hydrogen phosphate 0.92%, glucose 0.2%, ammonium sulphate 0.1%, magnesium sulphate 0.01%, sodium chloride 0.1%, pH 7.0 (adjusted with 5N NaOH or 5N HCl), malt agar: Bacto-malt extract (Difco) 2.0%, pH not adjusted. Cladospirone bisepoxide was applied at an end concentration of 50 μg per paper disc.

Germination Inhibition of Cress Seeds

5 seeds of *Lepidium sativum* arranged on paper discs (diameter: 20 mm) were exposed to different end concentrations of cladospirone bisepoxide dissolved in methanol (100 μg, 50 μg, 25 μg, 5 μg per paper disc). After 24 and 48 hours, the swelling, chlorophyll production and tip growth during germination were examined.

Fig. 3. UV spectrum of cladospirone bisepoxide: λ_{max}: 299 (ε=6,177); 313 (ε=4,655); 327 (ε=3,801).



Results

Investigation of Production Media

Among 25 different media examined for the production of cladospirone bisepoxide only 2 could be identified which produced detectable amounts after 96 hours. Medium M-1 consisting of 2.0%

defatted soybean meal and 2.0% mannitol gave a production titer of 731 mg/liter. Using fermentation medium M-2 which contained only 2.0% of oat meal, adjusted to pH 7.0, a titer of 236 mg/liter was obtained. The combination of the two media, resulting in medium M-3, led to a remarkable production increase to 1.16 g/liter already after a fermentation time of 48~72 hours.

In order to identify even more suitable carbon sources, mannitol was replaced by the ingredients shown in Table 1. All shake flask experiments were carried out at a temperature of 28°C and an agitation rate of 250 rpm. The amount of cladospirone bisepoxide was determined after 48 and 72 hours using HPLC as described above. As shown in Table 1 glucose and sucrose turned out to be the best carbon sources leading to titers of around 1.5 g/liter already after a cultivation time of only 48 hours. The replacement of mannitol by sucrose led to the production medium M-4 used for the fermentation on bioreactor scale.

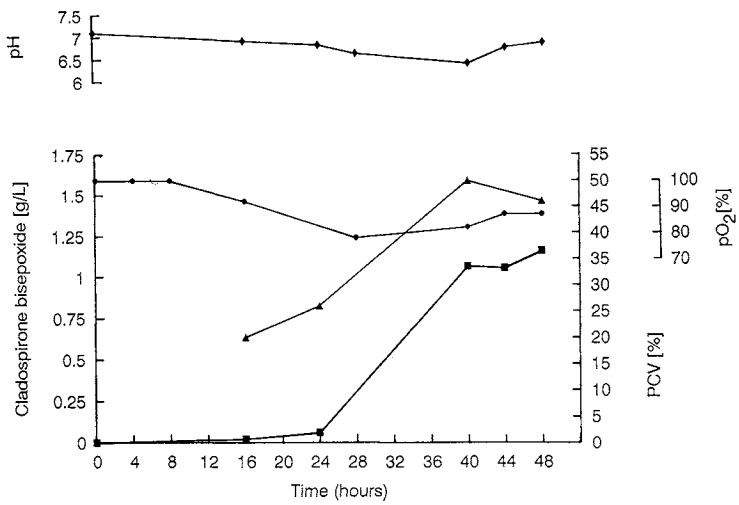
Fermentation on Bioreactor Scale

A characteristic time course of the cladospirone bisepoxide production is shown in Fig. 4. The logarithmic growth phase was terminated after 40 hours. Although a PCV of 50% was reached in this relatively short cultivation time, the oxygen partial pressure could be maintained at a sufficiently high level. The onset of cladospirone bisepoxide production took place after 16 hours. It is particularly worth mentioning that almost all the product is formed between 24 and 40 hours and that only very minor by-products are formed, indicating a highly efficient and tightly regulated biosynthesis. The maximum titer of 1.16 g/liter was reached after 48 hours. A prolongation of the fermentation time led to a drastic titer decrease (data not shown).

Table 1. Influence of various C-sources on the cladospirone bisepoxide production.

C-source (2.0%)	Cladospirone bisepoxide (mg/liter)	
	48 hours	72 hours
Potato starch	1,148	1,039
Sucrose	1,484	1,132
Lactose	535	371
Glycerol	287	82
Glucose	1,518	1,164
Oleic acid	792	1,153
Mannitol (control)	1,168	1,051

Fig. 4. Time course of cladospirone bisepoxide production in a 3.5 liter fermentor.
■ Cladospirone bisepoxide, ◆ pH, ● pO_2, ▲ PCV.



Antibiotic Activity

Cladospirone bisepoxide was tested against various microorganisms. It showed selective activity against yeasts, filamentous fungi and bacteria (Table 2).

Germination Inhibition

At a dose of 25 and 50 μg, the root tip growth was strongly reduced and the roots appeared remarkably intertwined. At higher concentrations, the growth was totally inhibited for a time of 48 hours. At lower concentrations of cladospirone bisepoxide, the seeds germinated like in the control experiment.

Table 2. Antibiotic activity pattern of cladospirone bisepoxide.

Test organism	Inhibition zone (mm)	Medium
Bacillus subtilis ETH 2016	36	Bss
Staphylococcus aureus ETH 2070	23	D.S.T
Sarcina lutea ATCC 9341	0	D.S.T
Escherichia coli ETH 2018	22	D.S.T
Pseudomonas aeruginosa ATCC 10145	0	D.S.T
Candida albicans ETH 6370	0	Mycophil
Saccharomyces cerevisiae ETH 108	24	Mycophil
Botrytis cinerea Tü 157	26	G 13
Piricularia oryzae AC 164	32	Mycophil
Mucor miehei Tü 284	0	HA
Paecilomyces variotii Tü 137	0	Mycophil

Discussion

Cladospirone bisepoxide (**1**) is a prominent representative of a rapidly growing group of fungal metabolites containing the spironaphthodioxine group and a highly substituted decaline moiety. The first representative of fungal metabolites belonging to this structural class was published only in the patent literature[2]. The antibiotic MK 3018 (**7**), isolated from strain *Tetraploa avistata* I 25 R (FERM P-9945) displayed a broad antibacterial activity. Based on physico-chemical data, the authors tentatively assigned structure **7** which is easily distinguished from (**1**) by its lack of the two epoxide functions. The diepoxins α (**3**), η (**4**), ζ (**5**) and σ (**6**) have been isolated from a non-sporulating fungus collected from a tree trunk growing in Panama[3]. A series of 6 closely related compounds isolated from the fermentation broth of *Natrassia mangiferae* has been disclosed in a recent patent application[4],*. The structurally similar monoepoxides called palmarumycins have been reported by a German group[7]. These metabolites are produced by two strains of the endophytic fungi *Coniothyrium palmarium* and *Coniothyrium* sp. A structurally related metabolite with a further modified 1,8-dihydroxy-naphthalene moiety has been isolated from the coprophilous fungus *Preussia isomera* Cain (CBS 415-82)[8]. This ascomycete colonizing cattle dung was detected and further investigated because of its antagonistic activity toward other coprophilous fungi *in vitro*. The complex structure (**8**) comprising seven ring systems could be elucidated by a X-ray crystallographic analysis of the methanol solvate. The absolute stereochemistry was assigned on the basis of the isolation of (−) regiolone (**9**), a decomposition product obtained by acid hydrolysis.

The novel metabolites of the spironaphtodioxine group display a surprising range of biological effects. In addition to inhibiting the growth of selected microorganisms and plants as reported in this paper, they also interfere with specific biochemical functions, such as the biosynthesis of interleukin-1β and with binding to some receptors (unpublished results). In order to define the structural features responsible for the various biological effects of cladospirone bisepoxide (**1**), the reactivity of the epoxide and enone functional groups has been explored by selective chemical transformations. The results of these investigations and the definite assignments of the structure and the absolute stereochemistry will be reported elsewhere.

Acknowledgments

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* Since the completion of this manuscript, two additional publications (refs. 5, 6) have appeared on this series of metabolites, which were extensively investigated as novel antitumor agents.

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