PH.D. THESIS

INVESTIGATION OF THE SECONDARY METABOLITE PRODUCTION OF FILAMENTOUS FUNGI

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INTRODUCTION

Members of the genus *Bipolaris* (*Ascomycota, Euascomycetes, Pleosporales, Pleosporaceae*) are imperfect filamentous fungi, which can be isolated from plant residues and soil and can be parasites of plants belonging to the family *Poaceae*. Phytopathogenic members of the genus caused several agricultural epidemics in the past century. The most typical symptoms of the infection are the spot blotch and the leaf blight, while some species can cause common root rot and crown rot. Teleomorph stages of the genus together with those of the closely related *Curvularia* are traditionally placed in the genus *Cochliobolus*.

Bipolaris species produce numerous secondary metabolites, such as sesquiterpenes (e.g. sorokinianin), diterpenes and sesterterpenes. The family of ophiobolins belongs to the latter group. Until now, 50 bioactive ophiobolin analogues have been described. Early studies identified them as phytotoxins. However, later examinations revealed their antimicrobial, bactericide, fungicide and nematicide effects, as well. Antifungal activities have been explained with the inhibition of the β-1,3-glucane synthase. Calmodulin antagonistic effect of ophiobolin A (OPA), the most extensively studied member of the ophiobolin family, was discovered in the 80's and constituted the basis of numerous investigations since then. OPA and some of its analogues, e.g. 6-epi-ophiobolin A (6-e-OPA), 3-anhydro-ophiobolin A (3-a-OPA) and ophiobolin I (OPI) proved to be cytotoxic against human cancer cell lines. Other ophiobolins, such as ophiobolin C (OPC), have HIV-1 integrase inhibitory effects.

Due to their diverse biological activities more and more increasing interest is tending towards the ophiobolins in the field of crop protection, medicine and biotechnology.

In this study, we aimed to expand the extant knowledge of the ophiobolins with special attention to the cultivation conditions, biological activities and stability. For several *Bipolaris* strains, OPA secretion kinetics was determined; then, an optimization of the cultivation conditions was carried out using the *B. oryzae* strain SZMC 24163 as the best producer. Using this strain, an effective semi-preparative methodology was developed for the purification of ophiobolin compounds. As the result of this purification procedure, a new ophiobolin analogue could be isolated. This compound was characterized by different structure determination procedures. Furthermore, OPA toxicity was characterized in different model systems and the stability of this compound was analyzed in various organic solvents.

OBJECTIVES

Members of the genus *Bipolaris* and related fungi (such as members of genera *Curvularia* and *Drechslera*) produce various sesterterpene-type secondary metabolites. Due to their diverse biological activities, they are considered as novel promising agents in biotechnological, medicinal and biocontrol developments. Recently several sesterterpene producing species have been described. However, detailed comparative studies on the producing organisms and the composition and quantity of the produced metabolites have not performed. Taking these facts into account, our aims were the followings:

1. Investigation of the ophiobolin production of different *Bipolaris*, *Curvularia* and *Drechslera* strains and identification of the outstanding OPA producers.
2. Development of a semi-preparative HPLC method for the purification of OPA and other ophiobolin compounds. Purification of ophiobolins from the strain, which proved to be the best producer in the previous studies. Mass spectrometric characterization and NMR identification of the purified compounds.
3. Determination of the optimal cultivation conditions of the strain with the best ophiobolin producing ability focusing on the inoculation method, the cultivation temperature and the applied carbon sources.
4. Analysis of the OPA stability in organic solvents and liquid chromatographic identification of the potential degradation products and determination of their production kinetics.
5. Investigation of the biological activities of the purified OPA on mammalian cells.

METHODS

Microbiological methods

- liquid phase fermentation
- optimization of fermentation

Separation technique methods

- extraction of ophiobolins
- analysis of ophiobolins using HPLC technique
- analysis of ophiobolins using LC-MS technique
- purification of ophiobolins using semi-preparative NP-HPLC technique
- purification of ophiobolins using semi-preparative RP-HPLC technique

Structure-related investigations

- structure determination using mass spectrometry
- structure determination using NMR technique
- stability investigations

Bioassays

- boar sperm motility assays
- examination of plasma membrane polarization on boar spermatozoa
- examination of the polarization of the mitochondrial-membrane
- examination of the effects of ophiobolins on tumor cell lines

RESULTS

1. Investigation of the ophiobolin production of different *Bipolaris*, *Curvularia* and *Drechslera* strains and identification of the outstanding OPA producers.

As a result of the secretion kinetics studies four types of kinetics were determined. In case of the first secretion pattern (type 1), OPA production showed one maximum value between the fifth and eight days of the cultivation, and after that, the amount of the produced OPA decreased to a constant level. In case of the second kinetic pattern (type 2), the amount

of the secreted OPA reached its maximum level between the fifth and seventh day followed by a dramatic decrease and was undetectable for the rest of the cultivation period in the ferment broth. For the third group (type 3), the OPA secretion profile showed two maximum points during the examination period, which were usually at the third and fifth days and at the ninth and tenth days, respectively. In the type 3 group, the values of maximums were approximately equal. Members of the type 1 group represented the largest proportion of the examined strains, while type 2 contained the 17% of the examined microbes. Only two strains belonged to the type 3 group and 30% of the examined strains were unable to produce OPA under the applied cultivation conditions (type 4).

During the characterization of OPA secretion kinetics we have successfully identified several strains possessing remarkable production capabilities, from which, the *B. oryzae* SZMC 24163 isolate has been selected for further preparative works.

2. Development of a semi-preparative HPLC method for the purification of OPA and other ophiobolin compounds. Purification of ophiobolins from the strain, which proved to be the best producer in the previous studies. Mass spectrometric characterization and NMR identification of the purified compounds.

Semi-preparative purification of ophiobolins was successfully executed in four separated batch using *B. oryzae* SZMC 24163 as the producer strain. In the first case, normal phase separation was applied as a first step in the purification after the extraction of the ophiobolins from the ferment broth. This was followed by a reversed phase separation. After each fractionation, the purity of the fractions was determined by the proper analytical method. Five potential ophiobolin analogues were purified from the first batch and they were successfully identified using mass spectrometry and NMR analyses. During the data evaluation, we demonstrated that all five purified compounds belonged to the family of ophiobolins, namely 6-epi-ophiobolin A, ophiobolin A, ophiobolin I, 3-anhydro-ophiobolin A and 3-anhydro-6-epi-ophiobolin A.

In case of the other three batches, only reversed phase separations were used in multiple consecutive steps. Besides the five known ophiobolins, five additional potential ophiobolin analogues were purified from these batches. Mass spectrometric examinations of these compounds were carried out. Based on these results, we can assume that they are also ophiobolin-type secondary metabolites. NMR examination of one of the potential ophiobolin

analogues proved that this compound is a new ophiobolin-type secondary metabolite, which has not been reported earlier.

3. **Determination of the optimal cultivation conditions of the strain with the best ophiobolin producing ability focusing on the inoculation method, the cultivation temperature and the applied carbon sources.**

Cultivation conditions of the selected strain were investigated to improve the ophiobolin production. Initially, the inoculation method was investigated. Two different inoculation procedures have been compared including the inoculation with conidial suspension and with mycelium in agar plugs. It was found that there is no significant difference between the two inoculation procedures, however inoculation with a suspension of 20 conidia/ml is considered to the most suitable to achieve the highest ophiobolin content. The effect of the cultivation temperature on the ophiobolin production was examined in liquid medium at four different temperatures. At the highest examined temperature (37 °C), there was no mycelial growth and any ophiobolin production. Cultivation at 28 °C resulted the highest amount of secreted ophiobolins, therefore this temperature was considered as optimal for the further experiments. To determine the optimal carbon source for ophiobolin production, ten different carbon sources were tested (i.e. glucose, fructose, maltose, sucrose, starch, cellobiose, ethanol, glycerin, mannitol and sodium-acetate). In these experiments, maltose proved to be the best carbon source, because its application resulted in the highest amount of secreted ophiobolins.

4. **Analysis of the OPA stability in organic solvents and liquid chromatographic identification of the potential degradation products and determination of their production kinetics**.

It is expedient to perform different bioassays using compounds with the highest possible purity. These compounds often meet with organic solvent during their purification process or their short-term storage. For bioassay studies, it is important to consider the degradation properties of the testing compounds. In the literature, only a few information can be found about the degradation of the different ophiobolin analogues and investigations on the degradation of this compound in organic solvents have not been performed. Therefore, our

objective was to test the stability of OPA in different organic solvents. Six different organic solvents (MeOH, EtOH, IPA, MeCN, EtOAc and DMSO) were involved in the study. OPA was primarily degraded in alcohols at high temperature. Two major degradation products (6-e-OPA and 3-a-6-e-OPA) could be successfully determined.

Furthermore, we observed that different degradation products were forming during the storage in different organic solvents.

5. Investigation of the biological activities of the purified OPA on mammalian cells.

The purified OPA was involved in biological activity assays. Bioactivity of OPA was investigated against sperm cells using boar semen and on three types of mammalian cell lines. OPA interfered sperm motility at low concentration. Furthermore, intense whipping movement was observed at 500 ng/ml concentration, this phenomenon has not been described until now. Furthermore, based on our results OPA was cytotoxic on MNA, FFL and PK-51 cells at the concentrations of <48.8, 48.8 and 48.8-97.6 ng/ml, respectively.

SUMMARY

1. *Bipolaris*, *Curvularia* and *Drechslera* strains involved in this study could be divided into four types of secretion kinetics. Strains with outstanding OPA secretion capabilities were determined.
2. A semi preparative HPLC method was developed for purification of ophiobolin analogues. Using this method, 10 potential ophiobolin analogues were purified.
3. The structures of 6 compounds were determined using NMR technique.
4. A new ophiobolin compound was described. It proved to be the 5-hydroxy derivate of OPA according to the applied NMR measurements.
5. Cultivation conditions of the *B. oryzae* SZMC 24163 strain stimulating the ophiobolin production were determined by testing the inoculation procedure, the cultivation temperature and the carbon source.
6. Decomposition kinetics of OPA in various organic solvents and the formation rates of the major degradation products were determined.
7. Cytotoxicity of OPA and four other ophiobolin analogues were investigated.

RESULTS SUMMARIZED IN THE PH. D. THESIS WERE PUBLISHED IN THE FOLLOWING ARTICLES:

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CUMULATIVE IMPACT FACTOR: 30.975