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# Paecilomide, a new acetylcholinesterase inhibitor from *Paecilomyces lilacinus*

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ABSTRACT

Fungi are some of the most important organisms in the production of bioactive secondary metabolites. This success is related to the advances in biotechnology and also to the possibility of working with techniques such as the "OSMAC" (one strain-many compounds) to achieve different fungal secondary metabolites profiles upon modifying the culturing conditions. Using this approach, the fungal species *Paecilomyces lilacinus* was cultivated in potato dextrose broth under 14 different fermentative conditions by adding the bacterium *Salmonella typhimurium* to the growing medium in order to provide biotic stress. *S. typhimurium* was added alive or after inactivation by autoclave or microwave irradiation in different stages of fungal growth. Extracts were prepared by liquid–liquid extraction using ethyl acetate, a medium polarity solvent in order to avoid extracting culturing media components. Production of fatty acids of relevance for the pharmaceutical and food industries was enhanced by the modified fermentative conditions and they were identified and quantified. The extracts were evaluated for acetylcholinesterase inhibition and the more active extract ($91 \pm 2.91\%$ inhibition) was prepared in large scale. From this active *P. lilacinus* extract, a novel pyridone alkaloid, named Paecilomide, was isolated and its structure was elucidated by modern nuclear magnetic resonance techniques and mass spectrometric analyses. Paecilomide (**1**) was also evaluated for acetylcholinesterase inhibition, presenting $57.5 \pm 5.50\%$ of acetylcholinesterase inhibition.



## Introduction

Fungi are important organisms in the production of bioactive secondary metabolites. Around 38% of the active compounds isolated until 2005 were of fungal origin (Bérdy 2005), and this context has not changed much in the late years. The success of fungal metabolites can be attributed to many factors, like the advances in the industrial production of biotechnological metabolites and the possibility of working with techniques such as the "OSMAC" (one strain-many compounds) (Bode et al. 2002). OSMAC is based on the premise that a single fungal species, upon submission to different cultivation conditions, can produce a great diversity of new bioactive molecules. Among the parameters that can be varied using OSMAC strategy, can be pointed the composition of culture medium, aeration, period of cultivation, pH, temperature and addition of agents to induce or inhibit the production of metabolites (Saleem et al. 2009; Bugni and Ireland 2004). Some stressing factors such as high osmotic levels, addition of a competitive microorganism in the medium (co-culturing), and water restraint have also been used in order to promote metabolic diversification in fungi (Cueto et al. 2001; Wang et al. 2011; Huang et al. 2011).

Many drugs currently in the market, possessing a variety of activities such as antitumor, immunosuppressants, antibiotics, hipocolesterolemic agents, antifungals, antiparasites, anti-inflammatory and enzyme inhibitors, were obtained from fungal metabolism (Bérdy 2005; Kingston 2011). Fungal metabolites have been shown their potential in the production of novel compounds (Zhang et al. 2011; Houghton et al. 2006) for treatment of Alzheimer's disease, a progressive and irreversible neurodegenerative disorder that leads to memory loss and cognitive disorders (Lima et al. 2009).

The symptoms of Alzheimer's disease are connected to the reduction of brain neurotransmitters, such as acetylcholine, noradrenalin and serotonin (Bryne 1998). Therefore, the treatment is based on the attempt to restore cholinergic function, using inhibitors of acetylcholinesterase (AChE), an enzyme that acts on acetylcholine degradation in the synaptic cleft (Lleo et al. 2006). Tacrine, Rivastigmine and Galantanine, AChE inhibitors available in the market, have a high cost, making necessary the search for new substances for treatment of Alzheimer's disease. Currently, this screening can be readily accomplished since there are some quick and sensitive screening bioassays to be used in the evaluation of acetylcholinesterase inhibitory effect caused by organic compounds (Ellmann 1961; Rhee et al. 2001).

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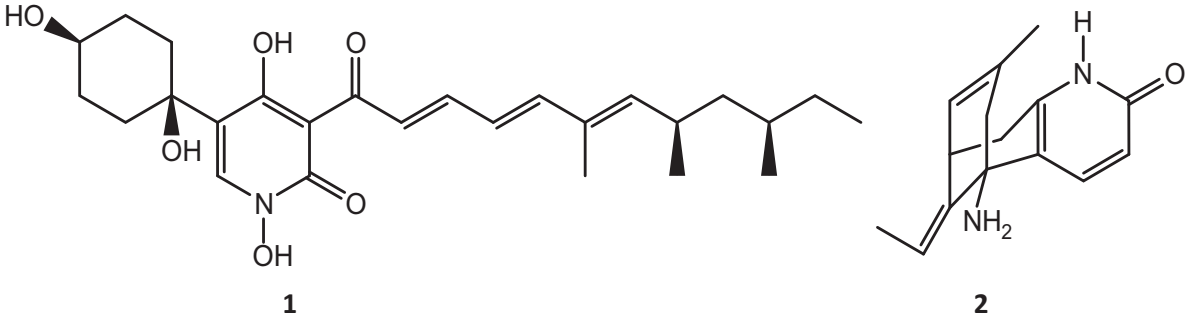


**Fig. 1.** Structures of Militarinone A (**1**) and Huperzine A (**2**).

The fungal species used in work was *Paecilomyces lilacinus*. *Paecilomyces* genus is divided into two sections, section *Paecilomyces* and section *Isarioidea*. The later contains mesophile members including *P. lilacinus* (Samson 1974). *P. lilacinus* 18S rRNA genes (rDNA) were sequenced and compared to other filamentous fungi species showing to be far differentiated from *Paecilomyces variotii*, a representative species from *Paecilomyces* genus (Wu et al. 2003). Species from *Paecilomyces* genus are capable of producing a variety of secondary metabolites of different chemical classes and with varied biological activities, such as cytotoxic, antibacterial, and immunostimulating agents (Kyong et al. 2001; Isaka et al. 2007; Liu et al. 2011; Xu et al. 2010). Neurotrophic pyridone alkaloids such as Militarenone A (**1**) have been reported from *Paecilomyces militaris* (Schmidt et al. 2002). These compounds possess structural resemblance with Huperzine A (**2**), a potent acetylcholinesterase inhibitor (Liu et al. 1986). Structures of compounds **1** and **2** can be found in Fig. 1.

No reports on the AChE inhibitors biosynthesis have been described for *P. lilacinus* under natural fermentation conditions. Therefore, OSMAC approach was exploited in order to create suitable stressing conditions with the aim of modulate the production of secondary metabolites with AChE inhibitory activity by *P. lilacinus*. From the existing stressing factors, such as increase of osmotic and atmospheric pressure, and decrease of nutrients availability, in this work, the stress was achieved by addition of bacterial genetic material in the culturing medium used to grow *P. lilacinus*. This strategy has been successfully employed to induce the production of bioactive secondary metabolites by other fungal species (Oh et al. 2005; Du et al. 2011). Several extracts were prepared, assayed and the most active extract for AChE inhibition was prepared in large sale. An active metabolite was isolated from this extract and identified. Fatty acids profiles were determined for all extracts obtained.

## Materials and methods

### *Source, maintenance and culturing conditions of the fungus P. lilacinus*

*P. lilacinus* was isolated from soil and it is deposited in the micro-organisms collection of the Biotechnology and Bioassays Laboratory (UFMG, MG, Brazil). *P. lilacinus* was maintained on potato dextrose agar culture medium (PDA) on a refrigerator (8 °C) (Schürmann et al. 2010). Prior to the experiments, the fungus was transferred to freshly prepared PDA and grown at room temperature (25 ± 3 °C). For pre-inoculum preparation, *P. lilacinus* was inoculated to Erlenmeyer flasks containing 200 mL of potato dextrose broth (PDB) and cultivated during seven days, under stirring (150 rpm), at 25 ± 3 °C. This procedure was performed to generate enough amount of biomass (pre-inoculum) to start the fungal cultivations.

### *Fermentations conditions and extracts preparation*

The experiments started with the inoculation of pre-inoculum into 14 Erlenmeyer flasks containing liquid medium (PDB, 200 mL/flask), after which the contents were extensively homogenized. Each flask was prepared to install the fungal growth in an odd condition on a medium containing *Salmonella typhimurium* in order to furnish biotic stress. *S. typhimurium* was added to the flasks containing *P. lilacinus* in two different concentrations (1 and 10 mL), in three different forms (alive, after microwave irradiation and after inactivation by autoclave) and in two different phases of *P. lilacinus* development (1st and 8th day of fungal growth). *P. lilacinus*–*S. typhimurium* were co-cultivated at room temperature (25 ± 3 °C), in static condition. Controls without addition of bacterial material were run in parallel. After a period of 21 days, the growth of *P. lilacinus* was interrupted by addition of EtOAc in the flasks. The culture media were individually filtered under vacuum through a filter paper, to separate the broths from the mycelia. The broths were exhaustively extracted with ethyl acetate on a separator funnel. This procedure was repeated three times. The mycelia have also been extracted with ethyl acetate, and both extracts (broth and mycelium) obtained in each of the 14 co-culturing conditions were combined, concentrated under vacuum and transferred to clean bottles.

### *Gas chromatography (GC) analytical conditions*

GC analysis was carried out on an HP5890 Gas Chromatograph equipped with Flame Ionization Detector (FID) to obtain the fatty acids profiles. A HP-INNOWax (HP) column (15 m × 0.25 mm) was used at the following temperature gradient: 150 °C, 1 min, 7 °C/min until 240 °C; injector (split of 1/50) at 250 °C and detector at 250 °C. Hydrogen was used as carrier gas (2.0 mL/min) and injection volume was 2.0 μL. Identification of compounds was made by comparison with SUPELCO37 fatty acid methyl esters (FAMEs) standard. The percentages of FAMEs were also compared with soybean oil (12.0 mg) hydrolyzed, methylated and analyzed under the same conditions.

### *Preparation of samples for GC analysis*

All extracts and some fractions from extract 5 (10.0 mg) were dissolved, on a 2.0 mL cryogenic tube, in 100 μL of a 1 mol/L potassium hydroxide solution (5%) in ethanol (95%). After vortex stirring for 10 s, the material was hydrolyzed on a microwave oven, during 5 min. After cooling, 400 μL of hydrochloric acid 20% (w/v), NaCl and 600 μL of ethyl acetate were added, stirred by 10 s and let to stand for 5 min. An aliquot of 300 μL of the organic layer was transferred to a tube and dried by evaporation, to obtain the free fatty acids (Segall et al. 2006). Free fatty acids were methylated with 100 μL $BF_3$/methanol (14%), heated for 10 min in water bath at 80 °C to