Journal of Photochemistry & Photobiology, B: Biology 178 (2018) 316–322

Contents lists available at ScienceDirect

Journal of Photochemistry & Photobiology, B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

Photoprotective potential of metabolites isolated from algae-associated fungi *Annulohypoxylon stygium*

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ARTICLE INFO

Keywords:
Annulohypoxylon stygium
Marine fungi
UV photoprotection
UV-filters
Phototoxicity potential
Secondary metabolites

ABSTRACT

Natural products, or secondary metabolites, obtained from fungal species associated with marine algae have been widely used in sunscreens due to their antioxidant activity and protective potential against solar radiation. The endophytic fungus isolated from *Bostrychia radicans* algae collected in the Rio Escuro mangrove, São Paulo State, Brazil, *Annulohypoxylon stygium* (Xylariaceae family) was studied to evaluate the photoprotective potential of its metabolites. The *Annulohypoxylon* genus can produce secondary metabolites with interesting cytotoxic, antibacterial and antioxidant properties and was never isolated before from a marine alga or had its metabolites studied for UV protection. The fungal culture (code As) extracted with dichloromethane: methanol (2:1) yielded 9 fractions (Asa to Asi) which were submitted to different chromatographic methodologies to obtain pure compounds, and to spectroscopic methodologies to elucidate their structures. Also, a screening was conducted to evaluate the qualitative production of the metabolites, besides the absorption in the UVA/UVB range, their photostability and phototoxicity potential using the 3T3 NRU phototoxicity test (OECD TG 432). This study led to the isolation of a novel compound, 3-benzylidene-2-methylhexahydropyrrolo [1,2-α] pyrazine-1,4-dione (**1**), from fractions Ase3 and Asf3; Ase1 was identified as 1-(1,3-Benzodioxol-5-yl)-1,2-propanediol (**2**), two metabolites were isolated as diastereomers (1*S*,2*R*)-1-phenyl-1,2-propanediol (**3**) from Asd2 and (1*R*,2*R*)-1-phenyl-1,2-propanediol (**4**) from Asd3, and Ase1 and 1,3-benzodioxole-5-methanol (**5**) from Asc1. The results obtained showed a great potential source of new molecules to be used as UVB filters in sunscreens, since substances **1–2** presented UVB absorption, had no phototoxic potential and were considered photostable. In conclusion, these compounds can be considered as a potential new class of molecules for photoprotection, since their photosafety and non-cytotoxicity were predicted using *in vitro* methods for topical use. Meanwhile, further efficacy assays shall be conducted for the establishment of their Sun Protection Factor (SPF). Also, this work provided new information concerning the metabolic profile of *A. stygium*, since it was possible to obtain two enantiomer compounds (**3**) and (**4**). One of them belonged to the same skeleton, but with a methylenedioxy moiety, showing the richest enzymatic pattern for this microorganism.

1. Introduction

The marine environment represents a global biodiversity and is the largest source of valuable and unique bioactive compounds from diverse marine organisms, most often with remarkable pharmacological potential such as anticancer, anti-inflammatory, antimalarial, antioxidant, antifungal, antibacterial, among others [1–3]. In this context, there has been an increasing interest in the utilization of products from novel bioprocessing technologies for the isolation of bioactive substances. Functional foods and nutraceuticals with antioxidant peptides isolated from the marine environment have become a topic of interest for pharmaceutical products and health foods [4]. Consequently, marine natural products (MNPs) have an increasingly important role in drug development as lead structures for bioinspired chemicals or directly as drugs [5]. An example is the extraordinary number of literature reports published in 2015 for MNPs, with 1220 citations, referring to compounds isolated from marine microorganisms and phytoplankton, green, brown and red algae, sponges, cnidarians among others, and from mangrove and other intertidal plants. It should be pointed out that the focus on 1340 new compounds published in 429 papers for 2015 is their relevant biological activity [6].

Oceans contain an extremely diversified wealth of organisms (algae,

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https://doi.org/10.1016/j.jphotobiol.2017.11.018
Received 14 September 2017; Received in revised form 31 October 2017; Accepted 12 November 2017
Available online 21 November 2017

crustaceans, shellfish, mollusks, and fish) [5]. Among them, marine microorganisms have become an important research area for drug discovery, as is the case for endophytic fungi that colonize the internal tissues of different species, such as sponges, jellyfish, and algae, which are mainly responsible for the production of bioactive secondary metabolites not found in terrestrial biodiversity [6,7]. Their relationship to the hosts is often described as a symbiotic association of mutualism, without causing any immediate overtly negative effects [8,9].

In addition, MNPs are secondary metabolites with a unique and sophisticated molecular structure which show diverse biological activities of medicinal relevance. However, the insufficient availability of MNPs needs extensive biological screenings and further target identification/mechanism-of-action studies [10]. Based on the biological potential of these microorganisms, nowadays it is of great interest to study and characterize them, mainly in terms of their metabolic profile (biosynthetic pathways) together with the biological activity associated with their potential. In recent years, with increased solar radiation on the earth, many organisms have been harmed, including those belonging to the marine environment. Thus, microorganisms, plants, and animals contain a variety of biosynthesized compounds that could be potential sunscreens [2,11]. A large number of compounds biosynthesized by marine algae and associated endophytic fungi have been studied in order to obtain new natural products that neutralize the damage caused by solar radiation. Some examples are: mycosporine produced by fungi, additional mycosporine-like amino acids found in algae, animals, and cyanobacteria, besides other UV absorption metabolites [12].

It is also important to mention that fungal studies are increasing, with 371 new compounds reported in 2015 compared to 318 in 2014 and 223 in 2013 [6]. The endophytic fungus identified as one of the species from the *Annulohypoxylon* genus, belongs to the Xylariaceae family (Ascomycete). This family comprises the type and largest family of Xylariales with at least 75 genera and a total of 800 or more species [13]. Also, it has long been considered to be part (Annulate) of the *Hypoxylon* genus. However, due to differences in morphological and taxonomic characteristics, it has been shown to be a new genus (*Annulohypoxylon*) [14,15].

In the marine environment, as in marine algae, environmental adversities related mainly to sun exposure, increase natural defenses against UV rays by producing secondary metabolites that can absorb/reflect sunlight, or act as antioxidants. Such molecules can be produced by the algae themselves or the associated endophytic fungi [16]. Only a few literature reports are related to secondary metabolites from this microorganism. Some of these compounds are species found in a terrestrial ecosystem: *A. cohaerens* (cohaerins AK), *A. multiforme* (multiformin A), *A. squamulosum* (diidrobenzofuran-2, 4-dione) and *A. stygium* (β-glucanases, pectinases, and xylanases) [17,20,21]. Mostly, they produce abundant pigments in their ascostromata (fruiting bodies) such as azaphilones that have been associated with a wide range of biological activities, including antioxidant activities [17–19] which may be of interest to the cosmetic industry for antiaging formulas or sunscreens.

Consequently, this study aimed to assess the photoprotective potential of fractions and substances isolated from the endophytic fungus *A. stygium*, which was isolated for the first time from the red seaweed *Bostrychia radicans*. We are also reporting here some enantiomers produced by this species, showing their metabolic diversity for the production of stereochemical compounds.

2. Methods

2.1. Strain

The specimen *Annulohypoxylon stygium* is an endophytic fungus isolated from the red seaweed *Bostrychia radicans*, collected from the Rio Escuro mangrove (Ubatuba city, São Paulo State, Brazil), in August 2007, and maintained in mineral oil at the Organic Chemistry Laboratory of the Marine Environment-NPPNS, Faculdade de Ciências Farmacêuticas de Ribeirão Preto - University of Sao Paulo, Brazil (FCFRP-USP). The strain was cultured on potato dextrose agar at 25 °C for seven days, and then ten plugs were inoculated into Erlenmeyer flasks with sterile rice and sea water at 25 °C. After 28 days of cultivation, the rice containing the mycelium was harvested and used as a sample for further extraction.

2.2. Extraction and Fractionation

Dried rice containing *A. stygium* mycelium was extracted twice with dichloromethane: methanol (CH_2Cl_2/MeOH (2:1)) at room temperature. The extract (45.00 g) was obtained by concentration under reduced pressure. The extract was analyzed by HPLC-DAD to determine the chromatographic profile and absorption was measured in the 200–400 nm range. From this extract, 20.00 g was fractionated by silica gel vacuum liquid chromatography (VLC) and eluted with a stepwise gradient of n-hexane/ethyl acetate/methanol, yielding nine fractions (Asa to Asi). Subsequently, fractions Asd and Ase (0.80 g and 0.65 g, respectively) were subjected to HPLC-DAD analysis using an RP-18 reverse phase analytical column with an increasing polarity gradient of acetonitrile-water (5–100%). The material was screened to determine absorption in the UV-A/UV-B range and qualitative analyses of the metabolites in the samples were performed. These two fractions were purified using a reverse phase semi-preparative column (RP-18) by HPLC-DAD and eluted with the same solvent gradient as described above.

2.3. Isolation and Identification of Compounds

Compounds **1–5** were obtained from fractions Asc1 (Compound **5**), Asd2 and Asd3 (Compounds **3** and **4**), Ase1 (Compound **2**), plus a novel compound of the class derived from 2,5-diketopiperazine from Ase3 and Asf3 (Compound **1**). Ase3 and Asf3 (photostable ones) were obtained on a semi-preparative scale from the organic extract of the fungi, submitted to one- and two-dimensional NMR spectroscopy, to spectrometric analyses using electrospray ionization (ESI) with time-of-flight (TOF), and to gas chromatography coupled to the mass spectrometer (GC–MS).

NMR spectra were recorded with a Bruker Avance III 600 HD spectrometer operating at 600 and 151 MHz for ^{1}H and ^{13}C NMR, respectively, and also with a Bruker Avance DRX 500 FT spectrometer operating at 500 and 125 MHz for ^{1}H and ^{13}C NMR, respectively. Mass data were obtained with a BRUKER® mass spectrometer model micro TOF II with an electron-spray ionization source (ESI) with type analyzer flight time (TOF) and NaTFA as the calibration solution. The sampler model was SIL-20ANT, communicator (CBM-20A) and oven (CTO-20A), using an RP-18 Supelco analytical column of 5 μm × 4.4 mm × 25 cm particle size, and monitoring at 270 nm with a Shimadzu Detector (SPM-20 A). The GC–MS analyses were conducted on a Shimadzu® QP 5000 analyzer using a 25 m × 0.25 mm × 0.25 μm D 3–5 column. Compounds analyzed by GC–MS were identified by comparison with the retention index (RI), calculated using alkanes from C9 to C22 (lot number 9673, Altech Associates, Inc. USA), an association of mass spectra with a computer databank (Wiley 7 and NIST 62), besides comparison with published data in the literature. Infrared analyses were recorded with a Nicolet-Protege 460 instrument. Chromatographic procedures were performed on silica gel 60 A (40–70 mesh) - MERCK®, using vacuum liquid chromatography (VLC). Analytical HPLC analyses were performed with a Shimadzu LC-6AD pump, using an RP-18 5 μm × 4.4 mm × 25 cm particle size column with a flow rate 1 mL min^{-1}, and monitoring at 200–400 nm with a Shimadzu detector. Semi-preparative HPLC separations were conducted with a Shimadzu LC-6AD pump using an RP-18 5 μm × 250 mm × 20 cm particle size column with a flow rate of 9 mL min^{-1} and monitoring at 320 nm with a Shimadzu detector.