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The *de novo* production of halogenated hydroquinone metabolites by the Andean-Patagonian white-rot fungus *Phylloporia boldo*

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Abstract

The production of halogenated hydroquinone metabolites such as drosophilin A, drosophilin A methyl ether and chloroneb was investigated in the Andean-Patagonian fungus *Phylloporia boldo*. These chlorinated compounds were detected in both fruiting bodies and living cultures. Gas chromatography–mass spectrometry (GC-MS) quantification of these molecules was performed in liquid media giving similar values in comparison to previous reports. We observed the concentration of drosophilin A, drosophilin A methyl ether and chloroneb increased in liquid culture supplemented with KCl. Furthermore, chlorinated hydroquinone compounds were not detected using liquid media supplemented with KBr. Instead, brominated aromatic molecules were observed and quantified by gas chromatography–mass spectrometry. We consider these results are relevant for the use of these halogenating microorganisms in biotransformation processes.

Key words – Drosophilin A – drosophilin A methyl ether – organohalogens

Introduction

In nature, halogenated organic compounds in terrestrial environments result from the decay of organic matter (Field 2016). Different organisms including fungi, bacteria, plants, sponges, insects and some mammals naturally produce organic halides (Häggblom & Bossert 2004). Several natural products contain a halogen in their structure and this motif plays a key role in their biological activity, with the most common halogens being bromine and chlorine (Agarwal et al. 2017). The incorporation of halogens in natural products is thought to be a strategy employed by microorganisms to enhance the biological activity of their secondary metabolites, increasing their chances of survival (Butler & Sandy 2009). Consequently, halogenated natural products exhibit a range of biological activities including, among others, cytotoxicity, antimicrobial or nematicide

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properties, and inhibition of the biosynthesis of chitin and melanin in ascomycetes (Anke & Weber 2006).

Fungi represent one of the largest groups of organisms (Hawksworth & Lücking 2017). Fungi-derived natural products are pharmaceutically prolific, having been developed into a number of important biological applications ranging from highly potent toxins to approved drugs (Hyde et al. 2019). Wood-decay fungi produce large amounts of chlorinated compounds under natural conditions (De Jong et al. 1994, Field et al. 1995, Teunissen et al. 1997, Verhagen et al. 1998, Garvie et al. 2015, Field 2016). Lignin is one of the most abundant biopolymers in terrestrial environments, functioning as a structural component in plants. It is thought that the chlorination of lignin takes place via the action of haloperoxidases that catalyze the formation of a Cl electrophile, hypochlorous acid (HOCl), which chlorinates lignin leading to its degradation (Field 2004). As a consequence of the catabolism of this biopolymer, the most common compounds produced by basidiomycetes are anisyl chloride and hydroquinone chloride methyl ethers (Teunissen & Field 1998). The biosynthesis of chlorinated metabolites is a highly dynamic process that is closely related to lignin degradation (Verhagen et al. 1996, De Jong & Field 1997, Field et al. 1997).

Chlorinated aromatic compounds have gathered significant attention since a number of them persist in the environment and display elevated levels of toxicity as well as bioaccumulation (Field & Wijnberg 2003, Hiebl et al. 2011). As a consequence, it is important to understand the fluxes as well as mechanisms ruling the biogeochemical chlorine cycle. In forest soils, chlorine is present in both organic and inorganic forms. Forests play an important role in the chlorine cycle and some cases, chlorine is found mainly bound to organic substrates (De Jong et al. 1994). The amount of organic chlorine is mainly mediated by basidiomycetes, which are known to actively produce chlorinated metabolites (Watling & Harper 1998, Harper 2000).

Tetrachloro-4-methoxyphenol, called drosophilin A (DA), was the first chlorinated metabolite isolated from basidiomycetes, specifically from *Parasola conopilea* (Kavanagh et al. 1952). The DA has subsequently been found in other fungi including *Phellinus fastuosus* (Singh & Rangaswami 1966), *Parasola plicatilis* (Bastian 1985), *Schizophyllum* sp. (Schwarz et al. 1992), *Agaricus arvensis*, *Bjerkandera adusta*, *Peniophora pseudopini* (Teunissen et al. 1997) and *Phylloporia ribis* (Lee et al. 2008). Furthermore, tetrachloro-1,4-dimethoxybenzene, also known as drosophilin A methyl ether (DAME), was first found in *Phellinus fastuosus* (Singh & Rangaswami 1966), and then later in *Phellinus yucatensis* (Hsu et al. 1971), *Phellinus robiniae* (Butruille & Dominguez 1972), *Agaricus bisporus* (Buss & Zimmer 1974), *Mycena megaspora* (Van Eijk 1975), *Peniophora pseudopini*, *Bjerkandera adusta* (Teunissen et al. 1997) and *Hypholoma fasciculare* (Verhagen et al. 1998).

While the study of tetrachlorinated hydroquinone metabolites has proven relevant for both biological and environmental reasons, most of the reports detecting and quantifying these types of molecules in fungi were released in the 1990's. This line of research was stagnant for almost two decades until 2015, when drosophilin A methyl ether was detected in the lignicolous basidiomycete *Phellinus badius* (Garvie et al. 2015).

Andean-Patagonian ecosystems in southern Chile display a high chemical and biological diversity. These ecosystems have unique microclimate and terrain conditions, which promote high levels of endemism (Donoso Zegers 1993). Mycological and chemical studies in these environments have been limited mainly due to challenges in accessibility and extreme weather conditions (Aqueveque et al. 2017). One of our research programs is dedicated to exploring fungal diversity as well as the chemistry to discover new fungal strains and molecules with relevant biological activities in Andean-Patagonian ecosystems. We have recently discovered a new fungus, *Phylloporia boldo* Rajchenb & Pildain (Hymenochaetales, Basidiomycota) (Rajchenberg et al. 2019) and during investigating the chemistry of this new strain, we found the presence of tetrachlorinated hydroquinone metabolites. To our knowledge, this work shows for the first time the presence and quantification of chloroneb, DA and DAME in an Andean-Patagonian fungus under different culture conditions. Furthermore, a shift in the synthesis of brominated compounds was observed using liquid media supplemented with inorganic bromine.

Materials & Methods

Strains

For this investigation, we employed the fungus *Phylloporia boldo* FQ1640. This strain was isolated in May 2016 from living stems of *Peumus boldus* located in the Santuario de la Naturaleza Península de Hualpén, Hualpén, Biobío, Chile. A duplicate was deposited at CIEFAP culture collection under number CIEFAPcc 532.

Culture conditions

Plate culture conditions were YMG agar, modified from Anke et al. (1995), containing 5 g L^{-1} granulated yeast extract for microbiology (Merck HGaA, Darmstadt, Germany), 10 g L^{-1} malt extract for microbiology (Merck HGaA, Darmstadt, Germany), 10 g L^{-1} D(+)-glucose monohydrate (Merck HGaA, Darmstadt, Germany) and 10 g L^{-1} granulated agar-agar (Merck HGaA, Darmstadt, Germany). Distilled water was used to prepare the cultures. Cultures were kept at 20°C in darkness and regularly subcultured. Our strain was preserved at 4°C. Liquid culture conditions are YMG medium (without agar) and were kept at room temperature (20-25°C) in a rotary shaker at 120 rpm for 3 weeks. Salts (KCl and KBr) were added (Spinnler et al. 1994) to reach a final concentration of 1 g L^{-1}.

Isolation and characterization of DAME

Some petri dish cultures displayed colourless needles on the surface of the mycelium. Preliminary identification by GC-MS suggested that it was the compound Drosophilin A Methyl Ether. Colourless DAME needles were manually extracted from the surface of the mycelial mat of Phylloporia boldo and then processed for NMR (Bruker Corporation, MA, USA) identification using deuterated chloroform. The spectroscopic data obtained matched the one reported for DAME (Song et al. 2008).

Extraction, sample preparation and quantification of halogenated hydroquinone metabolites using GC-MS

The preliminar fruiting body extract was performed using EtOAc from dried powdered basidiomes. Filtration and extraction of the liquid culture were performed as described (Swarts et al. 1996). After removal of EtOAc under reduced pressure, the remaining residue was redissolved in 1 mL of analytical grade EtOAc containing 4 µL at a final concentration of 5.8 mg mL^{-1} of 4-bromoanisole (Sigma-Aldrich, MO, USA) as the internal standard and then subjected to GC-MS analysis (Swarts et al. 1998).

GC-MS analysis

Analyses were performed using a GC-MS Shimadzu GC-17A (Shimadzu Corporation, Japan) equipped with a mass spectrometry detector MS-QP 5050A (Shimadzu Corporation, Japan) and helium as a gas carrier UHP 5.0 (99.999% purity) with a column flux of 1 ml min^{-1}. A column RTX-35 amine (30m x 0,25 mm x 0,50µm, Restek Corporation, PA, USA) was employed with a composition of 35% diphenyl / 65% dimethyl polysiloxane. The injector and the detector were both kept at 210°C. The method used was as follows: the oven was set at 100°C for 3 minutes and then heated up at a rate of 10°C min^{-1} until reaching 200°C, which was maintained for 17 minutes with a rate split of 1:1. Electron impact (EI) was used as the ionization method at 1.7 kV. Retention time of the method ranged from 4.1 to 29 minutes with a mass scan between 29 to 400 m/z. Structural identification of DA, DAME, chloroneb, 4-BR-2-MBA and 1,4-DB-2,5-DMB was achieved by comparing their mass spectra fragmentation patterns against NIST/EPA/NIH Mass Spectral Library (NIST 14).

Accession number

The DNA sequence for strains of *Phylloporia boldo* Rajchenb & Pildain were deposited in

GenBank (28S) under accession number MK193759.

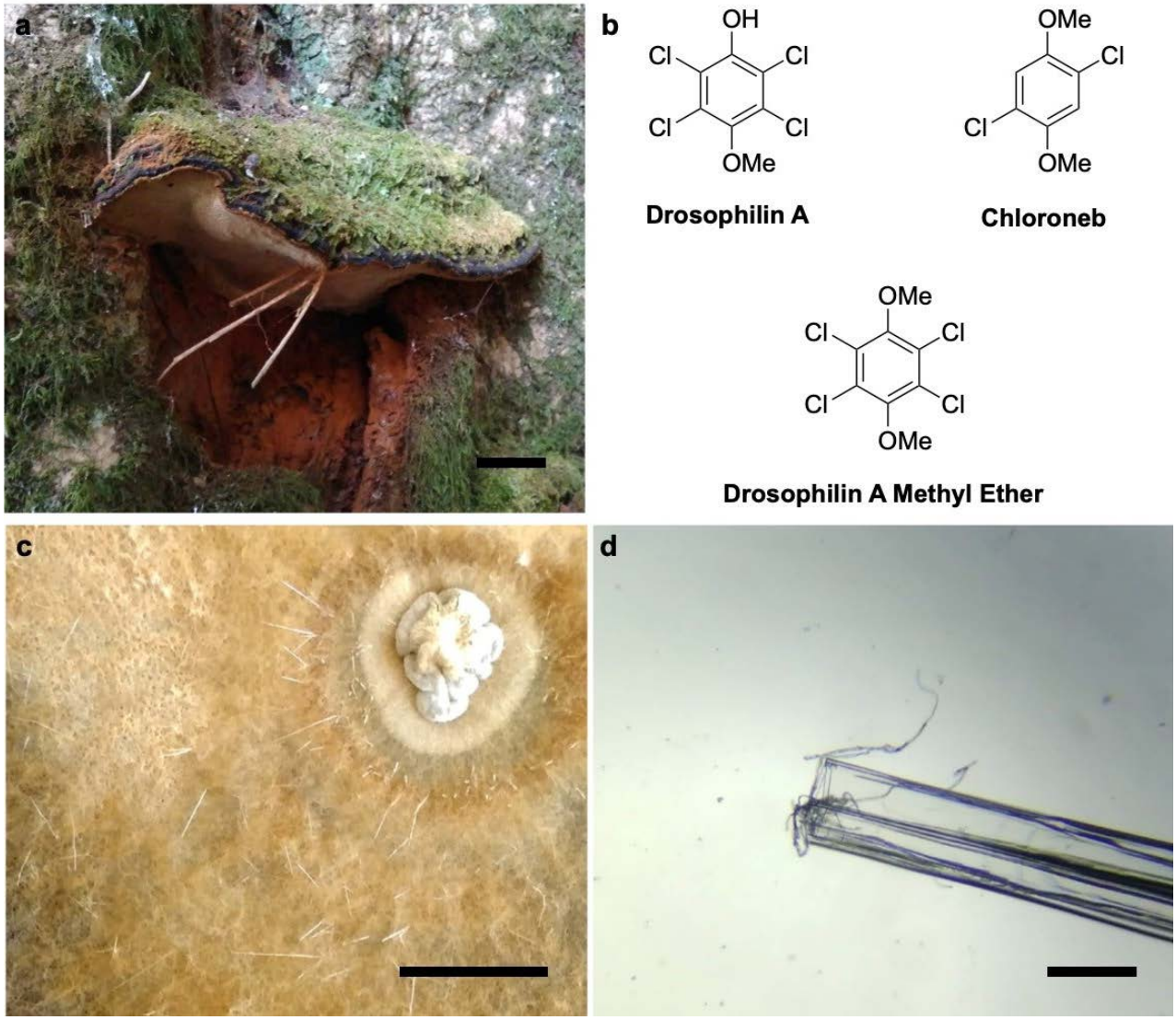

Fig. 1 – a Fruiting body of *Phylloporia boldo* Rajchenb & Pildain under sporulation growing over Peumus boldus. b Chemical structures of drosophilin A, drosophilin A methyl ether and chloroneb c Culture plate of *Phylloporia boldo* with superficial colourless needles of DAME. d DAME crystals. Scale bar: a = 1 cm, b = 1 cm, d = 100 μm.

Results

Detection of chloroneb, DA and DAME in fruiting body and plate culture of *Phylloporia boldo* Rajchenb & Pildain

Our investigation started by studying the fruiting bodies of *Phylloporia boldo* growing over *Peumus boldus* (Fig. 1a). GC-MS analysis of these samples revealed the presence of chlorinated hydroquinone metabolites such as DA, DAME and chloroneb (Fig. 1b). *Phylloporia boldo* was then cultured on a plate (Fig. 1c) and crystals of chlorinated hydroquinone metabolites (Fig. 1d) were observed, which were identified to be DAME by GC-MS analysis.

Quantification of halogenated hydroquinone metabolites by GC-MS of liquid culture of *Phylloporia boldo* under different conditions

Our efforts then focused on investigating halogenated metabolites in liquid culture of *Phylloporia boldo* (Fig. 2). We quantified these halo compounds without adding potassium halogen

salts to the liquid culture media. Under these conditions, after 3 weeks of growing, chloroneb, DAME and DA were detected at 0.04, 0.14 and 0.13 mg mL^{-1}, respectively (Fig. 2, Control). In the presence of KCl, liquid cultures of *Phylloporia boldo* rendered higher amounts of chloroneb, DAME and DA in comparison to control experiments; 0.08, 1.21 and 0.5 mg mL^{-1}, respectively (Fig. 2, KCl). We then supplemented the culture media with KBr instead of KCl. Interestingly, we did not observe any detectable amounts of chlorinated metabolites. However, we observed brominated molecules; 4-BR-2-MBA and 1,4-DB-2,5-DMB with a concentration of 0.27 and 0.38 mg mL^{-1} respectively.

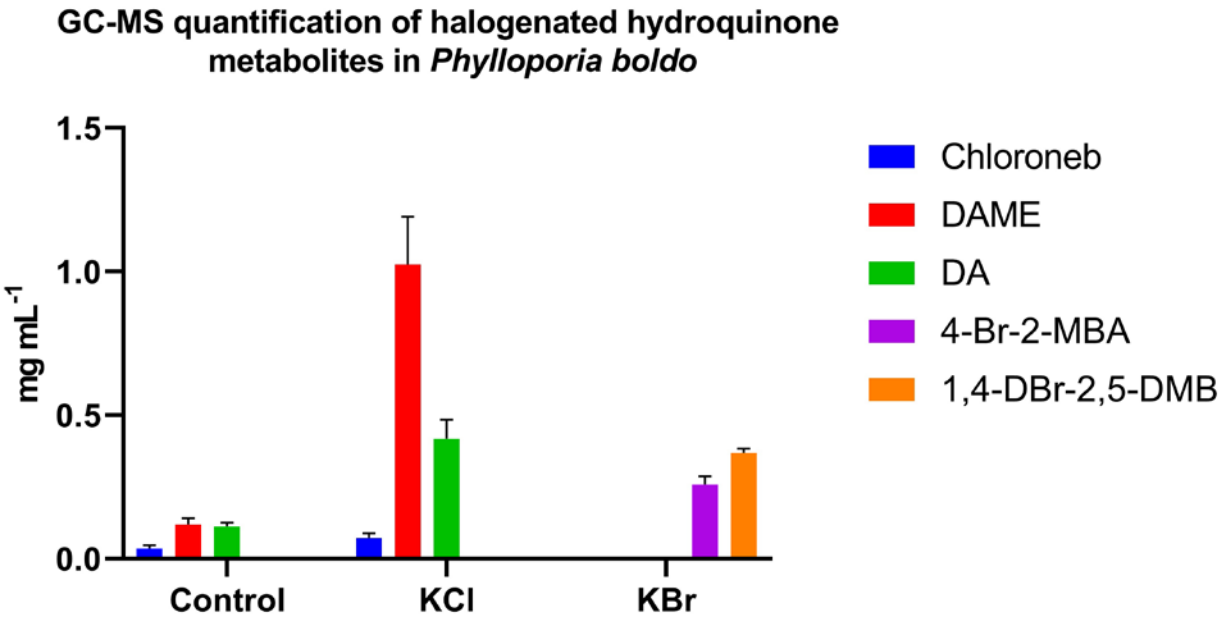

Fig. 2 – Quantification of halogenated hydroquinone compounds in liquid cultures of *Phylloporia boldo*. Results are shown as the average of triplicates with their standard deviation. 4-Br-2-MBA: (4-bromo-2-methoxyphenyl) methanol. 1,4-DBr-2,5-DMB: 1,4-dibromo-2,5-dimethoxybenzene. One-way ANOVA indicates there were significant differences between treatments for every evaluated compound ($p<0.0001$).

Discussion

Chlorinated hydroquinone metabolites play an important role in the biogeochemical chlorine cycle. Basidiomycetes are fungi that biosynthesize these chlorinated compounds mainly through the lignin degradation process. In the 1990's, a number of studies were reported revealing a range of fungal strains that synthetize chlorinated hydroquinone derivatives. Almost 20 years afterwards, a single work was published showing that the lignicolous basidiomycete *Phellinus badius* is able to produce DAME (Garvie et al. 2015).

Due to the special weather and terrain conditions, Andean-Patagonian environments in the south of Chile represent a unique ecosystem that promotes fungal diversity (Aqueveque et al. 2017). Despite this potential, Andean-Patagonian fungi have been poorly explored and their chemistry has received even less attention. We have recently reported a new taxon in the south in Chile, *Phylloporia boldo*, growing over *Peumus boldus*, an endemic Chilean tree (Rodríguez et al. 2018). The chemical properties of this fungus are unknown and preliminary efforts have just commenced. During these endeavours we detected chlorinated hydroquinone metabolites such as chloroneb, DA and DAME in this new fungus. We found crystals of DAME in fruiting bodies and cultures of *Phylloporia boldo*. The presence of crystals of halogenated hydroquinone metabolites has been reported before, in a single instance. While adding new strains to the arsenal of fungal producers of organochloride metabolites is important, we sought to investigate conditions aiming to

control the synthesis of these compounds by *Phylloporia boldo*. Thus, we evaluated the amount of chloroneb, DA and DAME in liquid cultures in the presence of inorganic halogen salts. Our controled experiments, without additional salts, revealed 0.04, 0.14 and 0.13 mg mL^{-1} of chloroneb, DAME and DA. These results are in accordance with the amount of these chlorinated metabolites observed in other fungal species (De Jong & Field 1997). In liquid media, YMG contains the chlorine and the carbon sources necessary to biosynthesize chloroneb, DA and DAME (Spinnler et al. 1994). Our efforts were to stimulate the production of chlorinated metabolites by adding KCl to the liquid media resulted in promising results; the amount of chloroneb, DAME and DA exceeded the values given by the control experiments (0.08, 1.21 and 0.5 mg mL^{-1}, respectively). There have been three reported strategies that promote the fungal biosynthesis of DA and DAME in liquid cultures: addition of 3,4-dichloroaniline, hydroquinone and the use of *in vitro* antagonism approaches (Teunissen et al. 1997). Nonetheless, to our knowledge, this is the first time that an enhancement of the production of DA and DAME has been accomplished by using KCl. Then, we attempted to shift the biosynthetic machinery to produce brominated rather than chlorinated compounds by adding KBr added to the liquid media instead of KCl. We observed exclusively brominated compounds; 4-BR-2-MBA and 1,4-DB-2,5-DMB with a concentration of 0.27 and 0.38 mg mL^{-1}, respectively. Chlorinated compounds such as chloroneb, DA and DAME were not detected under KBr conditions.

To our knowledge, this is the first report that shows the halogen shift in the biosynthesis of chloroneb, DA and DAME metabolites. The shift in the synthesis of brominated compounds using liquid media supplemented with KBr suggests that halogenating enzymes may have a high affinity for bromine (Peters & Spiteller 2006). While brominated organic molecules are widely present in marine environments, they are not very common in terrestrial environments. Thus, the ability of *Phylloporia boldo* to produce brominated hydroquinone derivatives is worth noting.

We consider these results are relevant for the use of these halogenating microorganisms for biotransformation processes. The functionalization of aromatic molecules is an important field of research, relevant to the pharmaceutical industry. Transforming a C-H bond into a C-Br or C-Cl bond selectively and efficiently could have an important application in chemical functionalization of pharmaceuticals, accelerating the development of drugs (Wagner et al. 2009, Abrams et al. 2018, Gandeepan et al. 2018). Thus, future efforts to further investigate the brominating ability of *Phylloporia boldo* using natural products as substrates, and testing the capability of incorporating iodine or fluorine into organic molecules are merited.

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