PHYTOCHEMICAL STUDY ON ECTOMYCORRHIZAL FUNGI *Cortinarius Magellanicus*: AN UNUSUAL BROMINATED SECONDARY METABOLITE ISOLATED

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ABSTRACT

Phytochemical analysis of the basidiomycete *Cortinarius magellanicus* (family Cortinariaceae) resulted in the isolation of eight secondary metabolites, where a brominated secondary metabolite was isolated for first time from a natural source. The structure elucidation of this compound was made using one- and two-dimensional NMR experiments, FT-IR, GC-MS, HRESIMS and chemical derivation. The disk diffusion assay of the brominated compound **4** and **8** revealed a low inhibition on Gram-positive and Gram-negative bacteria, respectively. No antifungal activities were detected for these compounds.

Keywords: Brominated saccharide, *Cortinarius magellanicus*, metabolite characterization, *Nothofagus* ectomycorrhizal fungi, phytochemical analysis.

1. INTRODUCTION

Fungi are organisms that colonize most habitats on the planet preferring dark and damp conditions where they collaborate with balance and contribute to the development of ecosystems [1]. Among the various fungi that exist globally can be found Cortinariaceae family. This family includes 33 genera, among which are found *Cortinarius* genus [2]. *Cortinarius* fungi are distributed globally but is considered the most ectomycorrhizal genus in *Nothofagus* forests of South America. In Chile, some specimens of this genus habit the austral zone and they are mainly found on plant remains in *Nothofagus* forests during the autumn-winter season [3]. In general, the different chemical studies of this genus show various structural models of secondaries metabolites, such as, β-carbolines, 1*H*-indole, quinolines, anthraquinones and terpenes [4–9]. Within the wide spectrum of secondary metabolites isolated, has shown the presence of volatile halogenated compounds. Also, from *Penicillium sp*. and *Bjerkander adusta* were identified, using GC-MS analysis, volatile chlorinated phenyl compounds from the apolar extract, which fulfil the role of growth hormone in the development of the fruit body [10,11]. In the same way, the study of *Boletopsis sp*. depicted the first polybrominated fungal from a terrestrial fungus [12]. On the other hand, substances isolated from fruiting bodies have been attributed bioactive activities such as AChE inhibitors, growth regulator, cytotoxics, antifungal, antioxidants, among others [9,13–15]. Genus *Cortinarius* has been studied extensively to know the nature of his secondary metabolites, biological applications, such others. From the selected fungi, only two investigations related to the identification of compounds are found. These studies identified the main secondary metabolites such as unsaturated fatty acids, saccharides, organic acids, and tocopherols. The aim of these investigations is related to the nutritional value of this edible mushroom [16,17]. Edible and medicinal mushrooms have been extensively studied. Within the main investigations, the isolation of secondary metabolites from *Armillaria Mellea*, *Pleurotus ostreatus*, *Suillus bellinii* and *Auricularia auriculajudae* resulted in obtaining aromatic sesquiterpenoids, dimeric pyronics, meroterpenoids and sterols, as well as the extract of *A. auriculajudae*. These compounds were presented antidepressant activity, antioxidant and immunomodulate activity, as precursor of the biosynthesis of collagen and cytotoxic and antitumoral activity, respectively [18–22]. This biological evidence associates the structure of the identified components and the health promoting properties of these species.

Regarding this work, the chemical study of *Cortinarius magellanicus* led the isolation of five secondary metabolites being one of them isolated from first time from natural sources.

2. EXPERIMENTAL SECTION

2.1 *General experimental procedures*

^{1}H-NMR spectra were registered dissolving the samples in $CDCl_3$ and D_2O at 400.13 MHz and 100.03 MHz for ^{13}C-NMR in a multidimensional Bruker Avance III HD-400 spectrometer. Chemical displacements are expressed as values relative to TMS as an internal standard. Bidimensional spectra were obtained using standard Bruker software. IR spectra were measured using KBr disk in the 500-4000 cm^{-1} region and were recorded on a FT-IR Thermo Nicolet Nexus 670 spectrophotometer, with 0.125 cm^{-1} spectral resolution. Solvents used in extractions and purification were dried and distilled by standard methods before use. Reagents were purchased from commercial suppliers were used without further purification. Purification took place in Isolera One using Biotage ® Ultra 10g HP-sphere 25 µm column. Exclusion chromatography was carried out using Sephadex LH-20. Mass spectra were taken in a CG Agilent 7890A with HP 5MS column coupled with an Agilent 5975C detector. Data acquisition was carried out in the full-scan mode and electron ionization mass spectra were recorded in the range of 35-650 (m/z). HRMS spectra were obtained on a Q Exactive Focus Hybrid quadripole-orbitrap mass spectrometer (Thermo Scientific, Waltham, MA, USA) using electrospray in the positive mode. The spray voltage was set to 3.5 kV; the sheath gas flow rate (N_2) to 50 units; the capillary temperature to 320 ◦C; the S lens RF level to 50; and the probe heater temperature to 425 °C. All data collected were compared with NIST 2005 database.

2.2 *Fungal material*

Fruiting bodies of *Cortinarius magellanicus* Speg. were collected in Tierra del Fuego Island, Tierra del Fuego and Antártica Chilena province, Chile, on *Nothofagus pumilio* forest during April of 2012. Live specimen was deposited in the fungary at Universidad de Concepción with the voucher RM 199.

2.3 *Total extract obtention of C. magellanicus*

The fruiting bodies of *C. magellanicus* were dried at 45 °C for 16 hours and grounded into a fine powder (4.85 g). The solid were extracted three times using methanol (20 mL) at room temperature. After filtering, the solvent was evaporated using vacuum at low temperature to form the extract.

2.4 *Acetylation of compounds 4-7*

In a 25 mL round flask loaded with a stirring bar, the mixture of solids obtained (350 mg) were added to 1 mL of anhydrous pyridine (1 mL). After complete dissolution, an excess of acetic anhydride was added, and the reaction mixture was stirred for 6 hours at room temperature. After complete derivatization of the components, the mixture was diluted with water and brine to later be extracted using dichloromethane (3 x 10 mL). The organic phase was dried using anhydrous sodium sulphate and the solvent was evaporated under reduced pressure. The acetylated fraction was adsorbed on a 1 g Biotage® SNAP Ultra samplet and eluted using a 10 g Biotage® Ultra column in an n-hexane/AcOEt gradient with increasing amounts of AcOEt, based on the parameters provided by the Accelerated Chromatographic Isolation (ACI).

2.5 *Deacetylation of compound 4*

In a 25 mL round flask loaded with a stirring bar, 35 mg of compound **4** were added to an 1% ethanolic $NaHCO_3$ solution. The reaction was stirred for 3 hours at room temperature and the solution was evaporated under reduced pressure to

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eliminate the excess of ethanol. The free compound **8** was used without further purification.

2.6 *Analysis by Gas Chromatography Mass Spectrometry (GC-MS)*

Identification of the isolated compounds were carried out using an Agilent Technologies 7890A gas chromatograph coupled to an Agilent 5975C detector. Helium was used as the carrier gas at an average flow rate of 1 mL/min. The capillary column used was an HP 5MS (30 m x 0.25 mm x 0.25 µm of thickness). Oven temperature was programmed as follows: 1 min at 80°C, raised to 200°C at 10°C/min, held for 3 min, ramped at 15°C/min up to 320°C and kept finally at 320°C for 8 min. The injection was made at 270°C and transfer line temperature was set at 250°C. Data acquisition was carried out in the full-scan mode and electron ionization mass spectra were recorded in the range of 35-550 (m/z).

2.7 *Antimicrobial activity*

The antibacterial activity was studied *in vitro* using the paper disk assay method (Cockerill et al. 2012). Test organisms included clinical isolates of *Staphylococcus aureus*, *Klebsiella granulomatis*, *Morganella morganii* and *Escherichia coli*. Bacterial strains were inoculated into Luria-Bertani broth and incubated at 37 °C. After 18 h of growth, the turbidity was adjusted using a 0.5 McFarland standard with sterile saline solution. Standardized bacterial cultures were individually swabbed on the surface of LB agar plates. Sterile filter paper disks (Whatman N°1, 0.6 mm diameter) were impregnated with the compounds, placed on the agar surface and incubated at 37 °C for 18 h. Disks impregnated with DMSO and streptomycin were included as negative and positive controls respectively. Plates were examined for zones of inhibition and the diameter of halos around disks was recorded in mm.

2.8 *Antifungal activity*

Antifungal activity was determined using the broth microdilution assay following the CLSI guidelines (Sheehan *et al.*, 2004). The fungi used were *Candida albicans* ATCC 10231, *Candida tropicalis* C131, *Cryptococcus neoformans* ATCC 32264, *Aspergillus niger* ATCC 9029, *Aspergillus flavus* ATCC 9170, *Aspergillus fumigatus* ATCC 26934, *Microsporum gypseu*m C 115, *Trichophyton rubrum* C113 and *Trichophyton mentagrophytes* ATCC 9972.

3. RESULTS AND DISCUSSION

3.1 *Isolation and characterization of secondary metabolites from C. magellanicus.*

From the fruiting body of ectomycorrhizal *Cortinarius magellanicus* Speg., two new compounds, 6-(acetoxymethyl)-2-bromotetrahydro-2*H*-pyran-3,4,5-triyl triacetate (**4**) and (2R,3R,4S,5S,6R)-2-bromo-6-(hydroxymethyl)tetrahydro-2H-pyran-3,4,5-triol (**8**), and six know compounds, ergosta-5,7,22-trien-3β-ol (**1**), 5α,8α-epidioxi-ergosta-6,22-dien-3β-ol (**2**), ergosta-7,22-dien-3β,5α,6β-triol (**3**), mannitol hexaacetate (**5**), trehalose octaacetate (**6**) and cellobiose octaacetate (**7**), were purified and characterised base on their NMR and MS data. Structure of the isolated compounds was found in Figure 1, 2 and 3, respectively.

Compounds **1-3** were elucidated using ^{1}H and ^{13}C NMR spectroscopy. In this context, spectroscopic data for *Cortinarius* genus suggest the presence of ergostane sterol in some extracts [23–25]. For our compounds, it possible identify the presence of six methyl group associated with the chemical shift of ergostane skeleton. In this sense, the ^{1}H and ^{13}C NMR for compounds 1-3 were correlated with literature data of typical ergostane sterols, suggesting the structure of these metabolites as ergosta-5,7,22-trien-3β-ol 1, 5α,8α-epidioxi-ergosta-6,22-dien-3β-ol 2 and ergosta-7,22-dien-3β,5α,6β-triol 3, respectively [23–26].

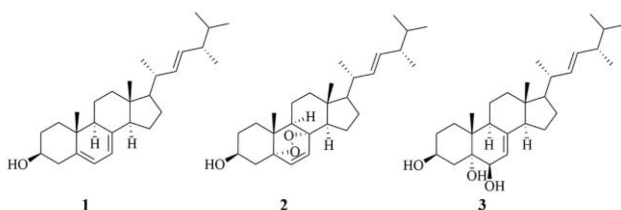

Figure 1. Sterols isolated for the fruiting body of *C. magellanicus.*

Compound **4** was obtained as white amorphous powder. DEPT spectrum suggests the presence of four methyl, one methylene, five methyne and four quaternary carbons of an ester. The ^{1}H NMR spectra shown a set of signals between δ_H 3.93 and 5.42 ppm, representing the characteristic core of six members belonging to a pyran ring [27,28]. The correct assignment of these protons and carbons were possible using COSY/HSQC correlation. Then, COSY spectra shown principal connections between H2 with H3, H3 with H2 and H4, H4 with H3 and H5, H5 with H4 and H6. The summary of these relationships can be found at Table S1 in supplementary material. Additionally, four signals were observed, between δ_H 1.99 and 2.04 ppm, and can be associated to a methyl in the acetoxy group. To confirm this, ^{13}C NMR spectrum reveal the presence of four methyl at δ_C 20.16, 20.24, 20.32 and 20.36 ppm and the corresponding carbonyl group at δ_C 169.64, 170.30, 169.75 and 169.50 ppm, respectively. Additionally, ^{13}C NMR data shown the characteristic methylene carbon of hexose at δ_C 62.23 ppm, where the HSQC spectrum connect that signal with the diastereotopic protons at δ_H 3.99 (*dd*, J=8.30, 5.05, 1H) and 4.17 ppm (*dd*, J=12.1, 5.05 Hz, 1H) assigned to assigned to Hα and Hβ, respectively. To confirm the relative configuration of **4**, NOESY spectrum shows intermolecular NOE interaction between H2/H3, H3/H5 and H4/H6. This information confirms the relative β configuration of H2. In this aim, considering the spectroscopic properties of D-glucose, the relative β orientation for H2, the chemical shift and the coupling constant between H2/H3 (δ_H 5.2 ppm (*d*, J=3.73 Hz, H2) for β-D-glucose) were consistent with spectroscopic assignment for compound **4** (δ_H 5.25 ppm (*d*, J=3.90 Hz, H2), Table 1 [28]. All the previous data and the confirmation of four acetoxy groups in **4,** suggest a different functional group in the anomeric carbon. To confirm the nature of this substitution, FT-IR spectra shown a band for carbonyl stretching at 1751 cm^{-1} and a particular band in 618 cm^{-1} associated to a bromine. On the other hand, just as expected for this class of compounds, ESI-MS analysis for compound **4** did not show a M+ or M+2 ion [29]. In order to confirm the presence of bromine, the characteristic signals at m/z 79 and 81 were positively observed. For this reason, the m/z 331 ion can be explained by the HBr loss from the original molecule. This confirmation suggests that the molecular formula of **4** was $C_{14}H_{19}O_9Br$. The HRESIMS of compound **4** shown a $[M – Br]^+$ ion at m/z 331.1021 (calcd for $C_{14}H_{19}O_9^+$, 331.1029), where this signal is consistent with the fragmentation of this compound in ESIMS, with a loss of bromine. Moreover, the other signals observed in the ESI-MS and HRESIMS spectra was consistent with the fragmentation of acetylated saccharide [29]. All the NMR data and GC-MS, in conjunction with the NIST 2005 database, confirms the structure of compound **4** as 6-(acetoxymethyl)-2-bromotetrahydro-2*H*-pyran-3,4,5-triyl triacetate. COSY and NOE interactions can be found at figure S16 in supplementary material.

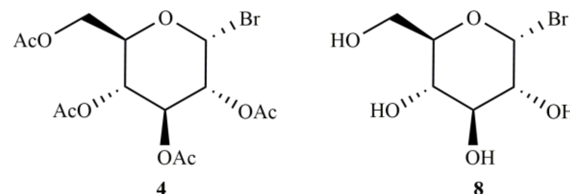

Figure 2. Structure proposed for compounds **4** and **8**.

To obtain the free compound (**8**), deacetylation of **4** was performed using ethanolic $NaHCO_3$ solution. Compound **8** was isolated as amorphous white solid and used without further purification, and its structure was confirmed using NMR experiments. The ^{13}C NMR spectrum showed 6 signals associated with 5 methines and 1 methylene. The absence of the set of signals in δ_C 20 and 169 ppm confirm the total hydrolysis of the four acetoxy group. Furthermore, the ^{1}H NMR spectra shown the presence of 7 signals associated to a characteristic methines in the pyran ring between δ_H 3.31 and 3.65 ppm for H3 to H6 and the anomeric hydrogen at δ_H 5.05 (*d*, J = 3.6 Hz, H-1) ppm. In addition, HRESIMS of compound **8** exhibit a $[M - Br]^+$ ion at m/z 163.0979 (calcd for $C_6H_{11}O_5$, 163.0606), who correspond to the free form of 3,4,5-triacetoxy-2-(acetoxymethyl)-2,3,4,5-tetrahydropyrylium ion. The figure 3 shows the proposed structures for compound **4** and **8**.

All these experimental observations of 1D and 2D NMR data, which confirms the proposed structure for compounds **4** and **8**, are summarized in table 1 (supplementary material). Therefore, compound **8** isolated from *C. magellanicus* is a novel (i.e. never previously reported) secondary metabolite. No reports of the natural isolation of this class of compound were found. Mainly, this brominated saccharide is used as an intermediate in *Koenigs–Knorr* reaction and industrial oligosaccharides and glycoconjugates synthesis [30,31].

Table 1. 1D and 2D NMR data for compound **4** and **8**.

	4				**8**	
N°	d_H	d_c	**DEPT**	^{1}H-^{1}H COSY	d_H	d_c
1	5.25 (*d*, J=3.90 Hz)	91.90	CH	3	5.05 (*d*, J=3.60 Hz)	93.15
2	4.96 *m*[a]	69.17	CH	2, 4	3.70 *m*[a]	72.10
3	5.42 (*t*, J=12.7, 10.3 Hz)	70.47	CH	3, 5	3.70 *m*[a]	72.42
4	4.98 *m*[a]	70.41	CH	4, 6	3.31 (*t*, J=9.30 Hz)	70.98
5	3.93 (*dd*, J=12.1, 10.3 Hz)	68.94	CH	5, 7	3.61 *m*[a]	69.91
6 α	3.99 (*dd*, J= 8.30, 5.05 Hz)	62.23	CH_2	6	3.50 (*dd*, J=9.60, 3.65 Hz)	60.44
6 β	4.17 (*dd*, J= 12.1, 5.05 Hz)				3.65 *m*[a]	
1’	2.01 *s*[b]	20.26[b]	CH_3	-	-	-
2’	2.04 *s*[b]	20.36[b]	CH_3	-	-	-
3’	2.03 *s*[b]	20.34[b]	CH_3	-	-	-
4’	1.99 *s*[b]	20.16[b]	CH_3	-	-	-
1’’	-	169.64[b]	C	-	-	-
2’’	-	170.30[b]	C	-	-	-
3’’	-	169.75[b]	C	-	-	-
4’’	-	169.50[b]	C	-	-	-

[a] *Signals are overlapped.* [b] *Interchangeable signal.* $CDCl_3$ was used in **4** and D_2O was used for **8**.

For the identification of mannitol hexaacetate (**5**), trehalose octaacetate (**6**) and cellobiose octaacetate (**7**), were characterised based on their MS data and contrasted with the NIST 2005 database.

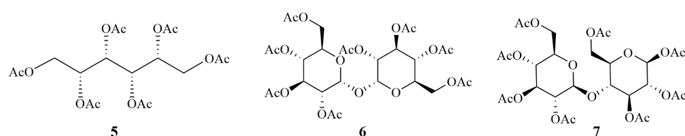

Figure 3. Secondary metabolites characterized using GC-MS analysis.

3.2 Antimicrobial activity.

Compounds **4** and **8** were evaluated the antimicrobial activity using the disk diffusion method on gram-positive and gram-negative bacteria as *Staphylococcus aureus*, *Klebsiella granulomatis*, *Morganella morganii* and *Escherichia coli*. The election of this biological activity was based in the presence of the bromine in our structures. In general, different type of compounds, with contain halogen, are more active than normal structures [32–34]. The test was evaluated using a concentration of 300 µg of compound per disk. Table 1 resume the inhibition zone observed for each compound. After diffusion test, the observed inhibition zone values for compound **4** shown low inhibition on Gram-positive bacteria while compound **8** inhibits slightly the Gram-negative bacteria in test conditions. This fact could be explained based on the type of cell wall that possess the Gram-positive and Gram-negative bacteria. Considering this feature, the hydrophobic nature of compound **1** facilitates the interactions with the cell wall in Gram-positive bacteria, what causes the cell death. In the case of compound **8,** the decrease in the permeability will cause no activity on these bacteria [35,36]. For Gram-negative bacteria, the lipophobic nature of the deacetylate compound **8** promotes the inhibition [37]. In general terms, these bacteria are resistant to compound **4** and **8** in test conditions used. For Antifungal activity, under test conditions, compounds **4** and **8** shown inactivity in fungal growth inhibition.

Table 2. Inhibition zones (mm) observed for the compounds (at 300 µ/disk) against bacteria using the disk diffusion method. DMSO and streptomicyn as negative and positive controls.

	Bacteria			
Compound	***K. granulomatis***	***S. aureus***	***E. coli***	***M. morganii***
4	-	3.5 ± 0.2	-	-
8	3.0 ± 0.1	-	-	2.5 ± 0.2
Streptomycin	n.t	17 ± 0.0	12 ± 0.0	n.t
DMSO	0	0	0	0

- No inhibition observed. **n.t** not tested

CONCLUSION

In conclusion, this study is the first report of the major metabolites secondary isolated from the fruiting body of ectomycorrhizal *Cortinarius magellanicus*. Further, from ethanolic extract was isolated, elucidated and characterized a brominated secondary metabolite for first time. The complete characterization and chemical derivation confirm the structure of this compound. In terms of the antimicrobial activity, Gram-negative and Gram-positive bacteria used are resistant against compounds **4** and **8**. This research opens the quest for new polar secondary metabolites from fungi, enables the search for other applications and the possible roles of these compound in the development or the survival of the fungi.

DISCLOSURE STATEMENT

No potential conflict of interest was reported by the authors.

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