ORIGINAL ARTICLE

Endophytic fungal compounds active against *Cryptococcus neoformans* and *C. gattii*

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Infections with *Cryptococcus* are invasive mycoses associated with significant morbidity and mortality, mainly in immunosuppressed patients. Several drugs have been introduced to combat these opportunistic infections. However, resistance of this organism to antifungal drugs has increased, causing difficulties in the treatment. The goal of this work was to evaluate the antifungal activity of ethanol extracts from endophytic fungi isolated from plants collected from different Brazilian ecosystems and to perform the fractionation of the most promising extract. Four-hundred fungal extracts were investigated by microdilution broth assays against *Cryptococcus neoformans* and *Cryptococcus gattii* at a concentration of 500 µg ml^{-1}. Among them, the extract of *Mycosphaerella* sp. UFMGCB 2032, an endophytic fungus isolated from the plant *Eugenia bimarginata* DC. (Myrtaceae) exhibited outstanding antifungal activity against *C. neoformans* and *C. gattii*, with MIC values of 31.2 µg ml^{-1} and 7.8 µg ml^{-1}, respectively. The fractionation of this extract using liquid–liquid partitioning and semi-preparative HPLC afforded two eicosanoic acids with antifungal activity, compound 1, (2S,3R,4R)-(E)-2-amino-3,4-dihydroxy-2-(hydroxymethyl)-14-oxoeicos-6,12-dienoic acid with MIC values ranging from 1.3–2.50 µg ml^{-1}, and compound 2, known as myriocin, with MIC values of 0.5 µg ml^{-1} against *C. neoformans* and *C. gattii*. These compounds are reported for the first time in the *Mycosphaerella* genus.
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INTRODUCTION

Cryptococcosis is a systemic invasive mycosis associated with significant morbidity and mortality with worldwide distribution of some species.[1,2] *Cryptococcus neoformans* and *Cryptococcus gattii* are the most important pathogenic species.[3] Cryptococcosis is an opportunistic infection that affects immunosuppressed patients, whereas *C. gattii* yeasts may also affect immunocompetent individuals.[1,4] Currently, the antifungal drugs used for cryptococcosis treatment are amphotericin B, 5-flucytosine and fluconazole. However, the pharmacological management of the disease is difficult because of the complexity of the infections and treatments and the reports of resistance and/or tolerance to the antifungals and their toxic effects.[5] The clinical syndromes and the treatment of infections caused by *C. neoformans* and *C. gattii* are very similar, but in some cases, a longer induction therapy may be required for a *C. gattii* infection. Moreover, this yeast has a propensity to promote massive inflammation. Mixed infections and drug resistance can occur, contributing to disease persistence and therapeutic failure.[6,7] It is therefore expected that new classes of antifungal agents can help overcome these problems.

Endophytic microorganisms can live in the intra-and/or intercellular environments of plant tissues without causing any apparent negative effect to the plants.[8] They are considered rich sources of bioactive natural products,[9,10] which have been considered promising alternatives for a variety of human health concerns.[10] Endophytic fungi associated with plants belonging to unique ecosystems, owing to environmental conditions, may produce specific secondary metabolites.[9] In this view, Brazil has a rich diversity of plant species that may be associated with endophytic fungi communities and which remains poorly explored for antifungal activity.[11] Ecosystems with unique characteristics such as microbial community, temperature, salinity and pH may contain many novel microorganisms species, including those able to produce compounds with undiscovered applications.[9,10]

The aim of the present study was to investigate the antifungal activity of crude ethanol extracts obtained from endophytic fungi associated with plants from different Brazilian ecosystems against the pathogenic yeasts *C. neoformans* and *C. gattii*, using a microbroth dilution susceptibility test and to perform the fractionation of the most promising extract. The present communication is concerned with the isolation and structural characterization of two eicosanoic-acid derivatives. These are antifungal compounds isolated and identified from the endophytic fungus *Mycosphaerella* sp., obtained from *Eugenia*

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bimarginata DC., a plant from the Myrtaceae family present in the Brazilian savannah.

MATERIALS AND METHODS

Sources of endophytic fungi

All plant samples were obtained from adult specimens collected in Brazil (savannah, Atlantic forest and Amazon rainforest). The plant identity, plant part collected, geographic coordinates and total number of endophytic fungi extracts evaluated are summarized in Table 1. Voucher specimens were deposited at the 'Instituto de Ciências Biológicas' herbarium (BHCB), 'Universidade Federal de Minas Gerais (ICB, UFMG)' and at the herbarium of 'Universidade Federal de Roraima', Brazil.

Isolation of the endophytic fungi

All samples of plant material were processed and the fungi was isolated as described previously.[12] In brief, the tissue samples were cut into pieces, placed in sterilized individual plastic bags and stored for <24 h at 10 °C prior to the isolation of endophytic fungi. Before surface sterilization, the leaves were cut into ~ 5-mm-long fragments using a flame-sterilized scalpel in a laminar flow hood to avoid air contamination of the tissues, and all samples were processed separately. The fragments were surface-sterilized by immersion in 2% Extran (1 min), 70% EtOH (1 min) and 2% sodium hypochlorite (3 min), which was followed by a wash with sterile distilled water (2 min). Afterwards, the fragments were plated on Petri plates containing potato dextrose agar (PDA, Himedia, Mumbai, India) supplemented with chloramphenicol (200 µg ml^{-1}) (Sigma, St Louis, MO, USA). The plates were incubated for up to 60 days at 25 °C, and the hyphal tip of each fungus growing out from the plant tissue was excised and transferred to a new PDA plate. After incubation at 25 °C for 10 days, culture purity was assessed using colony morphology. To test the effectiveness of the surface sterilization, 100 µl of the last water rinse was plated on PDA and incubated at 25 °C. Long-term samples of the filamentous fungal colonies were stored in cryotubes with 15% sterile glycerol at − 80 °C and in sterile distilled water at room temperature. All endophytic fungal isolates were deposited in the Culture Collection of Microorganisms and Cells at the 'Universidade Federal de Minas Gerais' under the code UFMGCB.

Fungi cultivation and extract preparation

Two-millimeter-diameter plugs of each endophytic fungus culture were inoculated into the centers of Petri dishes (90 mm diameter) containing 20 ml of PDA and incubated at 25 ± 2 °C for 15 days. The cultures were transferred to 50-ml vial tubes containing 40 ml of EtOH and macerated for 48 h at ambient temperature. The organic phase was decanted, and the solvent was removed in a vacuum centrifuge at 35 °C.[13]

Bioassay

Fungal isolates and inoculum. The antifungal activity of the extracts was evaluated using *C. neoformans* ATCC 24067 and *C. gattii* ATCC 24065. These yeast strains were stored frozen at − 80 °C, and the inocula were prepared according to document 7.1.[14] The starting inocula of *Cryptococcus* isolates were cultured onto Sabouraud dextrose agar twice for 48 h at 30 °C to ensure purity and viability. The inoculum was prepared by picking five distinct colonies that were suspended in 5 ml of sterile saline (0.85% saline), and the resulting suspension transmittance was adjusted with a spectrophotometer to reach the 1.0 McFarland turbidity standard at 530 nm wavelength. This procedure yielded a yeast stock suspension of 1–5 × 10^6 CFU ml^{-1}, from which was prepared a working suspension (1–5 × 10^5 CFU ml^{-1}) in sterile distilled water.

Screening for antifungal activity

All crude extracts were diluted to a final concentration of 500 µg ml^{-1} in RPMI 1640 (InLab, São Paulo, Brazil) supplemented with 2% glucose (Vetec, Rio de Janeiro, Brazil) and MOPS (Sigma). Amphotericin B (Sigma) at 2.0 µg ml^{-1} was used as a positive control. The wells (except the blank control) were inoculated with the yeasts to obtain a final inoculum of 0.5–2.5 × 10^5 CFU ml^{-1}. The microplates were incubated at 30 ± 2 °C for 48 h, after which 10 µl of a 5 mg ml^{-1} of MTT (3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Amresco, Solon, OH, USA) solution was added to each well. The plates were then incubated for 4 h and the absorbance measured at 570 nm using a microtitre plate spectrophotometer (VERSA*max*, Molecular Devices, Sunnyvale, CA, USA).[13,14] The data were collected and analyzed using the instrument software (Softmax, Molecular Devices). The percentages of inhibition (% inhib.) of the growth of the yeasts were calculated according to the following equation:

$$\%\text{Inhib.} = \frac{(\text{OD of negative control well} - \text{OD of sample tested})}{\text{OD of negative control well}} \times 100$$

Extracts with 75% inhibition of fungal growth against at least one *Cryptococcus* species were considered active. All susceptibility tests were performed in duplicate in three independent experiments.

Determination of MIC

The microdilution assays used to determinate the MIC were performed according to document 7.1 with modifications.[14] The concentrations of the samples ranged from 500 to 0.97 µg ml^{-1}. Fluconazole (Sigma) and amphotericin B (Sigma) were used as positive controls at concentrations of 0.125 to 64 µg ml^{-1} and 0.008^{-1} µg ml^{-1}, respectively. MIC end points were defined as the lowest concentration of samples that inhibited yeast growth (yellow well) with inhibition equal or superior to 90% of the growth in the control well.[14] All MIC values were obtained in duplicate in three independent experiments.

Molecular identification of the fungal isolate UFMGCB 2032

The internal transcribed spacer (ITS) region was amplified with the universal primers ITS1 and ITS4.[15] The amplification of the ITS region was performed as described previously.[16] Successfully amplified PCR products were purified using an ethanol/ethylenediaminetetraacetic acid precipitation protocol (Applied Biosystems, Foster City, CA, USA) and the sequencing reactions were performed at Myleus Biotechnology (www.myleus.com, Belo Horizonte, Brazil) on an ABI3130 automated sequencer (Applied Biosystems, Life Technologies, Foster City, CA, USA) using POP7 polymer and BigDye v3.1 with the same primers that were used in the amplification reactions. Quality control of individual sequences was performed by PHRED (http://asparagin.cenargen. embrapa.br/phph/), and only the sequences with at least 400 nucleotides and a PHRED quality score above 20 were considered. The consensus sequence was then generated by the CAP3 program (http://asparagin.cenargen.embrapa.br/ cgi-bin/phph/cap3.pl).[17] The consensus sequence (PHRED > 20) was subjected to phylogenetic inferences, which were estimated using MEGAVersion 5.0.[18]

Table 1 Host plant, data and number of extracts of endophytic fungi (EFE) assessed against *Cryptococcus* species

Host plant	Collected part	Habitat and GPS location	Total of EFE
Solanum cernuum	Leaves and stems	Atlantic forest (19°52′S, 43°58′W)	24
Eugenia bimarginata	Leaves	Savannah (19°52′S, 43°58′W)	7
Myrciaria floribunda	Leaves	Savannah (09°20′S, 49°58′W)	7
Alchornea castaneifolia	Leaves	Savannah (09°20′S, 49°58′W)	21
Stryphnodendron adstrigens	Leaves and stems	Savannah (21°05′S, 44°12′W) and (19°30′S, 43°54′W)	151
Carapa guianensis	Leaves	Amazon rainforest (0°57′02″N, 59°54′41″W)	194

The alignments were prepared including sequences of all relevant type strains or reference strains from culture collections deposited in GenBank to ensure accurate identification. The sequences were aligned using the MUSCLE method,[19] with manual adjustments for visual improvement where necessary. The Akaike information criterion was used to identify the most appropriate model of evolution for the data set. For ITS sequences, the data were estimated using the maximum likelihood method based on the general time reversible model, and a discrete gamma distribution was used to model evolutionary rate differences among sites (five categories (+G, parameter = 0.5758). The rate variation model allowed some sites to be evolutionarily invariable ((+I), 0.00% sites). The robustness of the trees was estimated by a bootstrap analysis[20] with 1000 replicates. The consensus sequence was submitted to GenBank and assigned an accession number from KF681521.

General experimental procedures

Thin layer chromatography analyses were conducted on pre-coated silica gel G-60/F$_{254}$ plates (0.25 mm, Merck, Darmstadt, Germany). Thin layer chromatography plates were eluted in a pre-saturated chamber using mixtures with different proportions of CHCl$_3$:MeOH:NH$_4$OH. The spots were visualized under visible and UV light (254 nm and 366 nm), and after heating the plate was sprayed with vanillin-H$_2$SO$_4$ reagent. Analytical reversed phase-HPLC analyses were run in a HPLC system (Shimadzu, Kyoto, Japan) equipped with a LC6AD pump and a SPD M-10 A VP diode-array detector. Analytical analyses were carried out on a Shim-pack C18 column (5 μm, 4.6 mm × 250 mm, internal diameter) eluted with a gradient of CH$_2$CN:H$_2$O at a flow rate of 1 ml min^{-1}. Semi-preparative purifications were done on a Shim-pack C18 column (5 μm, 20 × 250 mm, internal diameter) using mixtures of MeOH:H$_2$O or CH$_2$CN:H$_2$O as eluents. The flow rate was set at 8 ml min^{-1} and the effluent absorption measured at 220 nm on a Shimadzu SPD-10 A-UV detector (Shimadzu). Solvents were removed using a vacuum centrifuge (Thermo-Savant SPD SC250 Express, Holbrook, NY, USA). UV spectrum was recorded in MeOH on a Beckman DU Series 600 spectrophotometer (Beckman Instruments, Fullerton, CA, USA) and optical rotations were determined on a Modular Circular Polarimeter MCP 300 (Anton Paar, Ashland, VA, USA).

Large-scale cultivation and extraction procedures

Five 2-mm-diameter plugs of the culture of *Mycosphaerella* sp. were inoculated into each of 600 Petri dishes (90-mm diameter) containing 20 ml of PDA. The plates were incubated at 25 ± 2 °C for 15 days. The mycelium was separated from the medium with a spatula and both the medium and mycelium were extracted four times at 48 h intervals using ethyl acetate. The organic phases were grouped and concentrated on a rotary evaporator. The residual solvent was removed on a vacuum centrifuge at 40 °C to yield the crude extracts. These extracts were diluted in a MeOH:H$_2$O mixture (1:3) and successively partitioned between hexane (Hex), dichloromethane (DCM) and ethyl acetate (EtOAc). This procedure afforded the organic fractions denominated as hexane, dichloromethane and EtOAc, and Aqueous (Aq).

Isolation of antifungal compounds. An 800-mg portion of the EtOAc fraction residue was dissolved in MeOH:H$_2$O (1:1) and then centrifuged to remove suspended and insolubilized material. Nine aliquots containing ~ 100 mg of extract were injected into the semi-prep column. The column was eluted with a linear gradient of MeOH in H$_2$O starting at 10% and going to 50% in 30 min, from 50 to 95% in 20 min, from 95 to 100% in 1 min, and held at 100% for 17 min. Twenty-eight sub-fractions were obtained and tested in an antifungal assay. Active sub-fractions **13** (41 mg) and **14** (59 mg) were further fractionated by semi-preparative reversed phase-HPLC. The elution started with CH$_2$CN: H$_2$O 30% during 10 min, and the CH$_2$CN proportion increased to 50% in 14 min, 75% in 26 min, and then to 100% in 10 min and kept at 100% for 30 min. Sub-fraction **13** yielded 12 mg of compound **1**, whereas sub-fraction **14** afforded 21.5 mg of compound **2**.

Spectral data of the isolated compounds

One- and two-dimensional NMR experiments were run on a Bruker Avance 400 MHz spectrometer with standard pulse sequences using deuterated solvents containing 0.1% tetramethylsilane.

LC-DAD-MS analyses were performed using a Thermo Surveyor Plus chromatograph (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a Finnigan Surveyor PDA Plus diode-array detector. The analysis was carried out on a reverse phase column (Atlantis C18, Waters, Milford, USA, 3 μm particle diameter, 150 mm × 2.1 mm i.d.) using a linear gradient of CH$_2$CN:
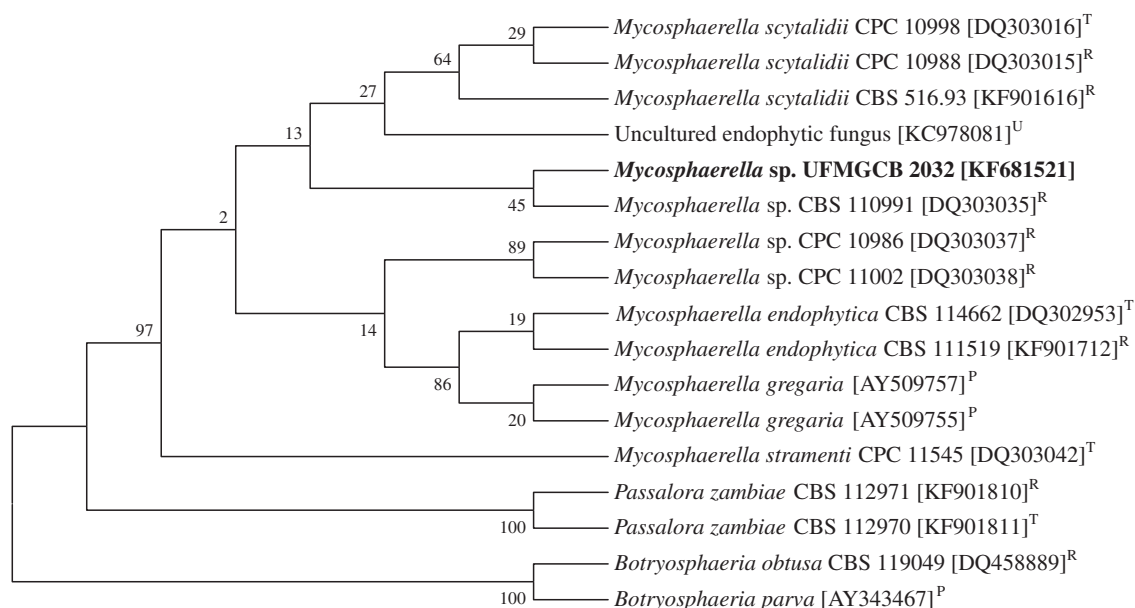

Figure 1 A consensus tree representing the phylogenetic analysis of the ITS region sequences of mycelia fungus (name and (accession number) in bold) isolates from *Eugenia bimarginata* DC. (Myrtaceae), in comparison with T (type strain), R (reference strains), P (from publications) or U (Unpublished) of the closest species (*Mycosphaerella* sp. (accession no.)), following BLAST analysis, deposited in the GenBank database. The tree was rooted with *Passalora zambiae*, *Botryosphaeria obtusa* and *Botryosphaeria parva* as the outgroup. Numbers at the branches are the bootstrap values of 1000 replications. There were a total of 726 positions in the final data set. The tree was constructed using MEGA software with the maximum composite likelihood method.

H_2O from 1 to 100% in 12.5 min. The effluent was entirely directed to the Bruker ETD-maXis quadrupole time-of-flight (Q-TOF) (Bruker Daltonics, Bremen, Germany) for ESI in the positive and negative ion modes. The mass detector was set to *the m/z* range 50–1500 Da. The instrument was operated under the following conditions: end plate offset, -500 V; capillary voltage, 4500 V; nebulizer pressure, 0.4 bar; dry gas (nitrogen) flow rate, $4.0 \, l \, min^{-1}$; dry temperature, 180 °C; in-source collision-induced dissociation energy, 25 eV; collision energy, 7 eV; ion cooler RF, 25 Vpp and transfer time, 45–49 µs.

RESULTS AND DISCUSSION
Screening for antifungal activity toward *Cryptococcus* species
Although endophytic fungi have been studied as producers of antifungal natural products, screenings against *Cryptococcus* as the main target using microdilution broth assays have been underexplored.[21,22] In our continued search for new compounds with antifungal activity from endophytic fungi,[11,13,23–25] attention was focused on the ethanol extracts of the endophytic fungi isolated from six plants growing in different Brazilian ecosystems (Table 1). They yielded 404 extracts that were tested at $500 \, \mu g \, ml^{-1}$ against *C. neoformans* and *C. gattii*. Four extracts (1.2% of isolates) showed >75% inhibition of fungal growth against at least one *Cryptococcus* species.

Table 2 MIC values of samples from *Mycosphaerella* sp. UFMGCB 2032 against *Cryptococcus neoformans* ATCC 24067 and *Cryptococcus gattii* ATCC 24065 using microdilution broth assays

Sample	*C. neoformans* MIC ($\mu g \, ml^{-1}$)	*C. gatti* MIC ($\mu g \, ml^{-1}$)
EtOAc crude extract	31.2	7.8
Hex fraction	500	62.5
DCM fraction	>500	>500
EtOAc fraction	31.2	7.8
Aq fraction	>500	>500
Compound **1**	1.3	2.5
Compound **2**	0.5	0.5
Amphotericin B	0.5	0.3
Fluconazole	4.0	4.0

The MIC value of the extract from UFMGCB 2032 ($7.8 \, \mu g \, ml^{-1}$) was close to that observed for fluconazole ($4.0 \, \mu g \, ml^{-1}$) in the assay with *C. gattii*. Fluconazole is a major antifungal agent used in the treatment of cryptococcosis because of its high efficacy, low toxicity and ability to be administered orally and parentally.[26] Therefore, the isolate UFMGCB 2032 was chosen to be submitted to chromatographic fractionation with the aim of identifying its antifungal compounds.

Identification of fungal isolate UFMGCB 2032
The identification of the fungal isolate UFMGCB 2032 was based on the analysis of the ITS region because it was not possible to obtain sporulating cultures with the specific conditions and culture media used to perform the morphological identification technique. The nucleotide sequence of UFMGCB 2032 did not show 100% alignment and 100% identity with any species deposited in GenBank. Other nuclear genomic loci such as β-tubulin and translation elongation factor 1-alpha were used to identify the isolate, but the amplification success rate failed to amplify these loci. The sequence of the fungus UFMGCB 2032 showed identity and coverage ranging from 99 to 98%, with sequences of uncultured endophytic and different species of *Mycosphaerella*; for this reason the fungal isolate was identified only at the genus level. The related sequences from the ITS region of the ribosomal RNA were retrieved from GenBank and were used for the phylogenetic analysis with MEGA 5 using the neighbor-joining method with 1000 bootstrap replicates. The phylogenetic analysis confirmed the distinct clustering of the isolate obtained in the study with *Mycosphaerella* species as the closest genotypes to the fungal isolate (Figure 1).

Chromatographic fractionation of *Mycosphaerella* sp. extract
The endophytic fungus *Mycosphaerella* sp. (UFMGCB 2032) was isolated from *E. bimarginata*, a plant from the Brazilian savannah. A literature search revealed that species of the *Eugenia* genus have some interesting properties such as antioxidant, anti-inflammatory, cytotoxic, anti-*Trypanosoma cruzi*, hypoglycemic and hypocholesterolemic activities.[27–30] In addition, antifungal activities have also been reported for a few species of the genus *Eugenia*. Clove oleoresin from *Eugenia caryophyllata* has shown fungicidal activity against *Candida albicans*, *Penicillium citrinum*, *Aspergillus niger* and
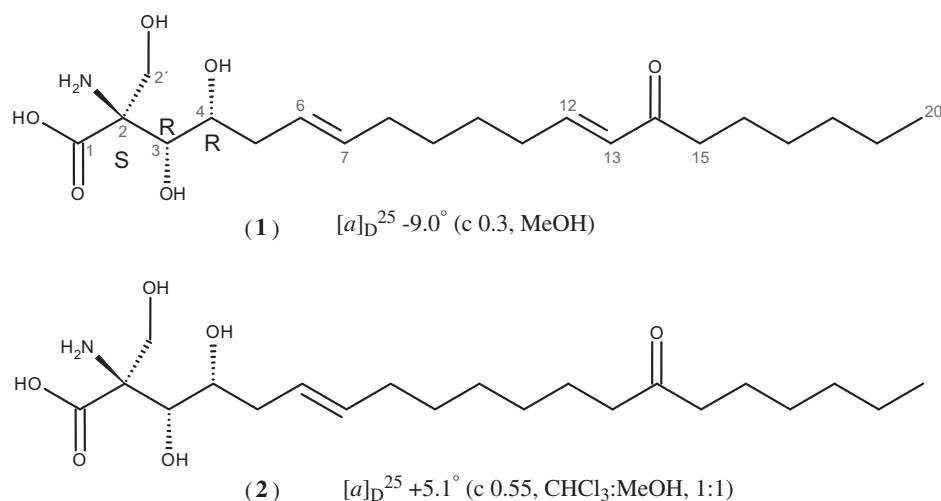

(**1**) $[a]_D^{25}$ -9.0° (c 0.3, MeOH)

(**2**) $[a]_D^{25}$ +5.1° (c 0.55, CHCl$_3$:MeOH, 1:1)

Figure 2 Structures of the compounds isolated from *Mycosphaerella sp* UFMGCB 2032. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

Table 3 Physicochemical properties of compound 1

Properties	Compound 1
Appearance	White powder
Molecular formula	$C_{21}H_{37}NO_6$
HRMS (ESI-QTOF) (*m/z*)	
Found	398.2533 $[M-H]^-$
Calcd. for $C_{21}H_{36}NO_6$	398.2543 for $[M-H]^-$
UV λ_{max}^{MeOH} nm (Log ε)	227 (2.55)
$[\alpha]_D^{25}$ (c 0.3 MeOH)	−9.0
Soluble	DMSO, methanol

Trichophyton mentagrophytes.[31] Essential oil from *Eugenia uniflora* L. was active toward several species of opportunistic pathogens belonging to the genera *Candida* (MIC 37.5–2.3 µg ml^{-1}) and *Cryptococcus*, including *C. gattii* (MIC 18.0–22.0 µg ml^{-1}) and *C. neoformans* (MIC 11.0 µg ml^{-1}). In this study, the major constituents of *Eugenia uniflora* L. essential oil, identified by analysis by GC-MS, are the sesquiterpenes atractylone (26.78%) and curzerene (17.96%).[27]

The ethyl acetate extract of *Mycosphaerella* sp. mycelium grown in PDA medium exhibited MIC values of 31.2 µg ml^{-1} against *C. neoformans* and 7.8 µg ml^{-1} against *C. gattii* (Table 2), respectively. The extract was subjected to a liquid–liquid partition sequence, and
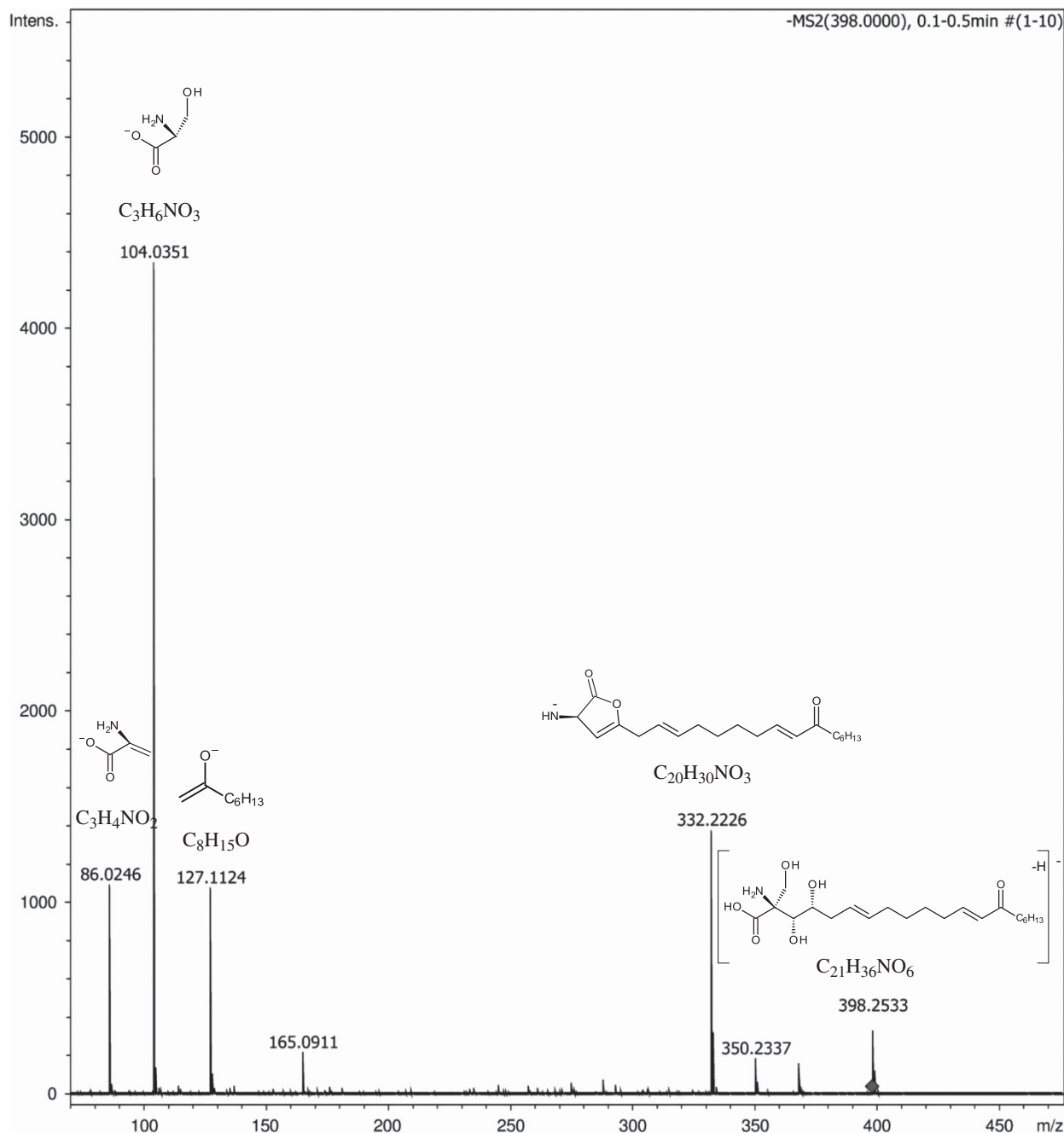

Figure 3 The ESI-(-)-LC-MS/MS spectra of compound **1** and proposal of the key fragments. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

this procedure afforded four fractions: hexane, dichloromethane, EtOAc and Aq. They were tested against the two *Cryptococcus* species and the results showed that the EtOAc fraction exhibited MIC values comparable to the crude extract (Table 2). In order to identify its active compounds, the EtOAc fraction was chromatographed twice by semi-preparative reverse phase HPLC to afford two active compounds (**1** and **2**) (Figure 2).

Structural elucidation of the active compounds isolated from *Mycosphaerella* sp.

The chemical structures of compounds **1** and **2** were established by analysis of NMR and mass spectrometric data and their comparison with the literature data. Compound **1** was isolated as an amorphous white solid with a specific rotation $[\alpha]_D^{25} - 9.0°$ (c 0.3 in MeOH) (Table 3). It gives a reddish-brown spot ($R_f = 0.45$; $CHCl_3$:MeOH: $NH_4OH/10$:6.25:0.25) after spraying the plates with vanillin-H_2SO_4. The negative high-resolution mass spectra (HRMS) (ESI-quadrupole time-of-flight (QTOF)) spectrum showed a quasi-molecular ion peak $[M - H]^-$ at m/z 398.2533 (calcd. 398.2543 for $C_{21}H_{36}NO_6$), consistent with the molecular formula $C_{21}H_{37}NO_6$. The mass spectrum obtained using tandem MS of compound **1** gave a base peak at m/z 104.0351($C_3H_6NO_3$) attributed to the fragment $[M - H - 294]^-$, and major fragments at m/z 332.2226 ($C_{20}H_{30}NO_3$), m/z 127.1124 ($C_8H_{15}O$) and m/z 86.0246 ($C_3H_4NO_2$). The proposal of the key fragments is shown in Figure 3. The UV spectrum of **1** showed λ_{max} at 227 nm. NMR spectra (one and two dimensional) showed ^1H signals

characteristic of a hydrocarbon chain at δ 2.54–1.21, corresponding to methylene carbons at δ 39.0–21.9 in the ^{13}C spectrum. Furthermore, the ^{13}C NMR spectrum of compound **1** showed peaks owing to four olefinic carbons resonating at δ 147.1 and 130.2, 131.6 and 127.0. These peaks were correlated to four olefinic protons at δ 6.83, 6.07, 5.43 and 5.35 in the ^1H NMR spectrum (Table 4). The geometry of the C_6/C_7 and C_{12}/C_{13} alkene bonds were *trans* (*E*), as evidenced by the large vicinal coupling constants ($J_{6-7} = 15.5$ Hz and $J_{12-13} = 16.0$ Hz). The ^{13}C NMR (DEPT) spectrum of **1** showed signals at δ 200.0 and at δ 169.9 of carbonyl carbons; the former was attributed to a ketone group and the latter to a carboxylic acid group. Correlation peaks between the H-13 (δ 6.07) and H-15 (δ 2.54) signals and the carbonyl at δ 200.0 in HMBC were observed and confirmed the location of the ketone group. HMBC correlations of the carboxylic acid carbonyl group at δ 169.9 confirmed its position at C-1 owing to cross peaks with H-2' (δ 3.56 and δ 3.69). The doublets at δ 3.69 (1H, d, J = 10.5, H-2'a) and δ 3.56 (1H, d, J = 10.5, H-2'b) were attributed to the geminal hydrogens of an oxygenated primary carbon. In addition, the ^1H NMR spectrum showed signals for two protons bound to the oxygenated tertiary carbons at δ 3.64 (1H, td, J 7.0, 1.5, H-4) and δ 3.61 (1H, br s, H-3). The values of the coupling constants of the signals at δ 3.64 and at δ 3.61 are similar to the values published for natural mycestericin A[32] and synthetic myriocin.[33,34] The ^1H–^1H COSY cross-peak between H-4 (δ 3.64) and H-5 (δ 2.09) suggests their vicinal location. The ^1H NMR and ^{13}C NMR spectral data of compound **1** exhibited the characteristic signals of aliphatic chains

Table 4 NMR data for compounds 1 and 2 (DMSO-d6) at 400/100 MHz, δ in ppm and J in Hz

	Compound 1				Compound 2			
Position	δ ^{13}C (DEPT)	δ ^1H (J)	^1H-^1H COSY	HMBC (H→ C)	^{13}C (DEPT)	δ ^1H (J)	^1H-^1H COSY	HMBC (H→ C)
1	169.9 (C)			H-2'a, H-2'b	169.7 (C)			H-2'a, H-2'b
2	67.6 (C)			H-2', H-3	67.8 (C)			
3	69.3 (CH)	3.61 (*br s*)			69.3 (CH)	3.61 (*br s*)		
3-OH		5.02 (*br s*)a				5.02 (*br s*)a	H-3	
4	70.7 (CH)	3.64 (*td, J* 7.0, 1.5)	H-5		70.7 (CH)	3.64 (*td, J* 7.0, 1.5)	H-5	
5	37.5 (CH$_2$)	a: 2.09 (*ddd, J* 7.0)	H-4, H-6, H-7		37.5 (CH$_2$)	a: 2.09 (*ddd, J* 7.0)	H-5b, H-6	H-4, H-6, H-7
		b: 2.16 (*m*)				b: 2.15 (*ddd, J* 7.0)	H-5a	
6	131.6 (CH)	5.43 (*dt, J* 15.5, 6.5)	H-5, H-7	H-8	131.9 (CH)	5.42 (*dt, J* 15.3, 6.5)	H-7, H-8	H-8
7	127.0 (CH)	5.35 (*dt, J* 15.5, 6.5)	H-6, H-8	H-8	126.7 (CH)	5.34 (*dt, J* 15.4, 6.5)	H-5, H-6, H-8	H-8
8	31.8 (CH$_2$)	1.96 (*br q, J* 6.5)	H-7	H-6, H-7	32.1 (CH$_2$)	1.93 (*q, J* 6.7)	H-6, H-10	H-6, H-7
9	28.4 (CH$_2$)	1.34 (*br q, J* 7.5)	H-8	H-8, H-10	28.4 (CH$_2$)b	1.31–1.29 (*m*)a	H-8	
10	27.0 (CH$_2$)	1.42 (*br q*,7.5)	H-11	H-8, H-9, H-11	28.8 (CH$_2$)	1.28–1.27 (*m*)a	H-8	H-8
11	31.6 (CH$_2$)	2.19 (*br q, J* 7.0)	H-10, H-12, H-13	H-12, H-13	28.3 (CH$_2$)b	1.26–1.25 (*m*)a	H-10	H-9, H-12
12	147.1 (CH)	6.83 (*br dt, J* 16.0, 7.0)	H-11, H-13	H-11	23.2 (CH$_2$)	1.44 (*qui, J* 7.0)	H-13	H-11, H-13
13	130.2 (CH)	6.07 (*dt, J* 16.0, 2.0)	H-11, H-12	H-11	41.8 (CH$_2$)	2.38 (*t, J* 7.2)	H-12	H-12
14	200.0 (C)	–		H-12, H-13, H-15	210.58 (C)			H-12, H-13, H-15, H-16
15	39.0 (CH$_2$)	2.54 (*t, J* 7.3)	H-16	H-16	41.8 (CH$_2$)	2.38 (*t, J* 7.2)	H-16	H-16
16	23.7 (CH$_2$)	1.47 (*br quin, J* 7.0)	H-15, H-18	H-15	23.2 (CH$_2$)	1.44 (*qui, J* 7.1)	H-15, H-17	H-15
17	28.2 (CH$_2$)	1.23–1.21 (*m*)a		H-15, H-16	28.5 (CH$_2$)b	1.22–1.20 (*m*)a	H-16	H-15, H-16
18	31.0 (CH$_2$)	1.26–1.24 (*m*)a	H-16, H-20	H-16, H-19, H-20	31.1 (CH$_2$)	1.24–1.23 (*m*)a	H-20	H-19, H-20
19	21.9 (CH$_2$)	1.30–1.27 (*m*)a	H-20	H-17, H-20	21.9 (CH$_2$)	1.26–1.25 (*m*)a	H-20	H-17, H-20
20	13.9 (CH$_3$)	0.85 (*t, J* 7.0)	H-18, H-19	H-18	13.9 (CH$_3$)	0.85 (*t, J* 7.0)	H-19	H-18
2'a	62.4 (CH$_2$)	a: 3.69 (*d, J* 10.5)	H-2'b		62.4 (CH$_2$)	a: 3.70 (*d, J* 10.5)	H-2'b	
2'b		b: 3.56 (*d, J* 10.5)	H-2'a			b: 3.58 (*d, J* 10.5)	H-2'a	

a^1H NMR signals assignment was rationalized by HSQC contour map and Perch NMR software calculations.
bInterchangeable signals.

(δ 1.21–2.54/ δ 21.9–39.0) and signals due to a terminal methyl group were observed at δ 0.85 (1H, t, $J = 7.0$ Hz, H-20) and at δ 13.9 (C-20). The similarity between shifts and coupling constants values found in the ^{13}C NMR and ^{1}H spectra of both **1** and **2**, at C-1 to C-5 and H-3 to H-5 proton signals, respectively, suggested that these two structural moieties are identical in **1** and **2**. However, the comparison between the $[\alpha]_D$ values for **1** (−9.0°, c 0.3, MeOH) and myriocin (+5.1°, c 0.18, MeOH)[35] shows that they are comparable in magnitude but with opposite signs. This reversal effect on the $[\alpha]$ sign of **1** in comparison with **2** could result from

conformational effects due the double bond at C-12.[36] Fujita *et al.*[37] also reported that the compound 6,7-dihydromyriocin, obtained by the hydrogenation of myriocin, is a levorotatory compound ($[\alpha]_D$ − 6.5°, c 0.108, MeOH). This sign reversal effect was also found when comparing $[\alpha]_D$ values for myriocin[35] and 14-*epi*-mycestericin A.[38] Thus, the relative stereochemistries at C-2, C-3 and C-4 of **1** were assigned as 2S, 3R and 4R, respectively. On the basis of these observations, compound **1** was identified as (2S,3R,4R)-(E)-2-amino-3,4-dihydroxy-2-(hydroxymethyl)-14-oxoeicos-6,12-dienoic acid, as shown in Figure 2.
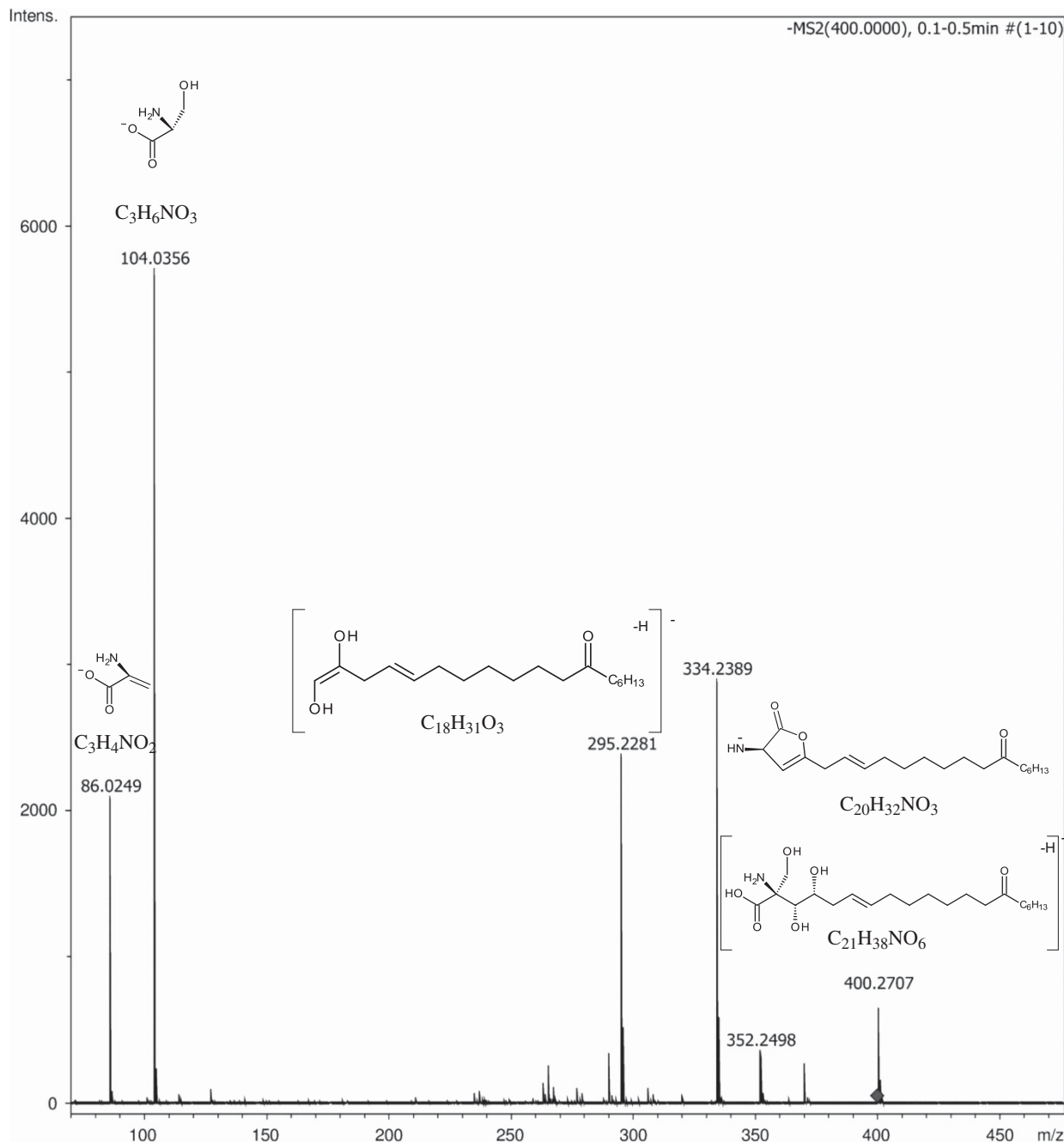

Figure 4 The ESI-(-)-LC-MS/MS spectra of compound **2** and proposal of the key fragments. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

Compound **2** was isolated as an amorphous white solid, and the UV spectrum exhibited no absorption maximum above 200 nm. It showed a positive specific rotation ($[\alpha]_D^{25}$ +5.1°, c 0.55 in CHCl$_3$:MeOH 1:1). The negative HRMS (ESI-QTOF) spectrum of compound **2** showed a *quasi*-molecular ion peak $[M-H]^-$ at *m/z* 400.2707 (calcd. 400.2699 for C$_{21}$H$_{38}$NO$_6$), consistent with the molecular formula C$_{21}$H$_{39}$NO$_6$. The mass spectrum obtained using tandem MS of compound **2** showed an ion peak $[M-H-296]^-$ at *m/z* 104.0356 (base peak, C$_3$H$_6$NO$_3$), and the main fragments at *m/z* 334.2389 (C$_{20}$H$_{32}$NO$_3$), *m/z* 295.2281 (C$_{18}$H$_{31}$O$_3$) and *m/z* 86.0249 (C$_3$H$_4$NO$_2$), respectively. The proposal of the key fragments is shown in Figure 4. Analysis of the NMR and HRMS spectra of compound **2** showed that its main difference from compound **1** is the absence of a double bond at C$_{12}$–C$_{13}$. Furthermore, the respective ^{1}H NMR spectrum signals for H-2' (a and b hydroxymethylene protons), as well as the $[\alpha]_D$ value reported for the isomeric compound 2-*epi*-myriocin,[39] differ significantly from those found for compound **2**. On the basis of these observations, **2** was identified as (2*S*,3*R*,4*R*)-(*E*)-2-amino-3,4-dihydroxy-2-(hydroxymethyl)-14-oxoeicos-6-enoic acid, known as (+)-myriocin (**2**) and shown in Figure 2. All ^{1}H and ^{13}C NMR (one and two dimensional) data for **1** and **2** can be seen in Table 4.

Antifungal activity of isolated compounds

Mycosphaerella spp. are important phytopathogenic species, causing diseases in wheat, banana, *Eucalyptus* species and other crops.[40,41] Bioactive compounds were isolated from other species of *Mycosphaerella*. Asteromine, a 6,6′-binaphtho α-pyrone isolated from *M. asteroma*, has antifungal activity against *Ustilago maydis*, *Ophiostoma ulmi* and *Geotrichum candidum*.[42] Cercosporin, a perylenequinone metabolite of polyketide origin, was isolated from *Mycosphaerella* sp. nov. strain F2140 associated with the foliage of the plant *Psychotria horizontalis* (Rubiaceae). Cercosporin exhibits high cytotoxicity activity (MCF-7, IC$_{50}$ 4.68 μM) and also non-selective anti-protozoan activity against *Plasmodium falciparum* (IC$_{50}$ 1.03 μM), *Leishmania donovani* (IC$_{50}$ 0.46 μM) and *Trypanosoma cruzi* (IC$_{50}$ 1.08 μM).[43]

In the present work we show that eicosanoic acids, the oxidized derivatives of C20-fatty acids produced by *Mycosphaerella* sp. UFMGCB 2032, possess antifungal activity. Compound **1** exhibited MIC values of 1.3 and 2.5 μg ml^{-1} against *C. neoformans* and *C. gattii*, respectively. Compound **2** showed a MIC value of 0.5 μg ml^{-1} to both species of *Cryptococcus* tested. The antifungal activity of natural product analogs from the eicosanoid family is still little explored, specifically against *Cryptococcus* pathogenic species. According to the literature, there are no previous reports of the isolation of compound **1**, whereas compound **2** (myriocin) was first described by Kluepfel *et al.*[44] and later isolated from *Myriococcum albomyces*, *Melanconis flavovirens*, *Isaria sinclairii* and *Paecilomyces variotii* ATCC 74097.[45] They are reported herein for the first time as natural products from this endophytic species. Myriocin is a serine palmitoyltransferase inhibitor[46] and has immunosuppressant activity. This metabolite also exhibits antifungal properties against several pathogens (*Alternaria solani*, *C. albicans*, *C. parapsilosis*, *C. pseudotropicalis*, *C. tropicalis*, *Ceratocytis ulmi*, *Cryptococcus laurentii*, *Fusarium oxysporum*, *Rhizomucor miehei* and *Streptomyces* spp.[44,47]

These two eicosanoic-acid compounds exhibited higher antifungal activities against *Cryptococcus* species than the crude original extract and fractions. Considering the antifungal activity of the compounds demonstrated in this work, their *in vivo* efficacy in a murine model of *Cryptococcus* infection should be assessed.

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