Thieme

Laccaridione C, a Bioactive Polyketide from the Fungus *Montagnula* sp.

OPEN ACCESS

Authors

Celso Almeida[1], Thomas Andrew Mackenzie[2], Víctor González-Menéndez[1], Nuria de Pedro[2], Ignacio Pérez-Victoria[3], Gloria Crespo[3], Jesús Martín[3], Olga Genilloud[1], Francisca Vicente[2], Bastien Cautain[2], Fernando Reyes[3]

Affiliations

1   Fundación MEDINA, Microbiology, Armilla, Spain
2   Fundación MEDINA, Screening and Target Validation, Armilla, Spain
3   Fundación MEDINA, Chemistry, Armilla, Spain

Correspondence

Dr. Fernando Reyes
Fundación MEDINA
Avenida del Conocimiento 34
18016 Armilla, Granada
Spain
Tel : + 34 958993965, Fax : + 34 958846710
fernando.reyes@medinaandalucia.es

Dr. Celso Almeida
Fundación MEDINA
Avenida del Conocimiento 34
18016 Armilla, Granada
Spain
celsoguerreiro@gmail.com

Supporting Information for this article available online at http://www.thieme-connect.de/products.

ABSTRACT

The new polyketide laccaridione C **(1)** was obtained by bioassay-guided isolation of organic extracts of the fungal strain CF-223743, isolated from dung collected in a forest of Grand Comoros Island. Its structure was established using spectroscopic methods, namely HRMS and 1D and 2D NMR. The new compound was tested against seven cancer cell lines, evidencing effective activity against melanoma (A2058) with an IC_{50} of 13.2 μM, and an increased activity against breast cancer (MCF-7) with an IC_{50} of 3.7 μM. The strain CF-223743 was taxonomically identified as *Montagnula* sp. based on ITS/28S analysis

Introduction

Natural products are important sources of anticancer lead molecules [1]. More than 60 % of the current compounds with antineoplastic activity were originally isolated as natural products or are natural product derivatives, and microbial metabolites are among the most important of these chemotherapeutic agents [2]. Melanoma is a malignancy of pigment producing cells (melanocytes), which are located primarily in the skin, but are also found in the ears, gastrointestinal tract, eyes, oral and genital mucosa, and lep-

tomeninges [3]. Malignant melanoma is the most aggressive form of skin cancer and accounts for about 3 % of all cases of malignant tumors. Its incidence is increasing worldwide, and it is becoming resistant to current therapeutic agents [4]. In a recent report, 30 *in vivo* and *in vitro* natural active principles were reviewed for their pharmacological effects on migration and/or metastasis of melanoma cells, mapping the mechanisms of action for these underexploited properties. They were described as acting mainly through their antagonistic effects upon the TNF-*α* and EP2 receptors or the

Almeida C et al. Laccaridione C, a Bioactive … Planta Med Int Open 2019; 6: e57–e62

e57

suppression of several protein kinases involved in metastatic pathways such as RAS, PI3K, ERK, and FAK. Some were able to reduce the level of mesenchymal biomarkers such as N-cadherin and/or elevate the expression of other molecules such as E-cadherin [5].

Herein, we report the isolation and structural elucidation of the cytotoxic polyketide laccaridione C (**1**) (▸ **Fig. 1**) obtained from culture broths of the fungal strain CF-223743 after fractionation of ethyl methyl ketone extracts based on melanoma targeted activity. A phylogenetic placement of the strain CF-223743, based on its ITS/28S, identified the fungus as a species of the genus *Montagnula*, an ascomycete of the order Pleosporales (▸ **Fig. 2**). The related polyketides laccaridione A (**2**), laccaridione B (**3**) [6], and leptosphaerodione (**4**) [7] were also detected as minor components in the extract after LC-DAD-HRMS-based dereplication [8].

Results and Discussion

Compound **1** was isolated from the ethyl methyl ketone extract of a culture of *Montagnula* sp. (CF-223743) by melanoma activity-guided fractionation on SP207SS resin followed by repeated semipreparative HPLC. The molecular formula of **1** was inferred to be $C_{21}H_{22}O_6$ by accurate mass measurements (ESI-TOF, see **Supporting Information**) (m/z 371.1489, $[M+H]^+$, calcd. for $C_{21}H_{23}O_6^+$, 371.1479), which translates into 11 degrees of unsaturation. The structure of **1** was established by 1D and 2D NMR spectroscopy (▸ **Table 1** and **Supporting Information**). The ^1H NMR and HSQC spectra displayed signals corresponding to four sp^2 methines, two aliphatic methines, one of them oxygenated, one aliphatic methylene, a methoxy group, and three aliphatic methyl groups with singlet, doublet, and triplet multiplicities. Two hydroxy protons were also observed, with one of them corresponding to a phenol involved in intramolecular hydrogen bonding according to its sharp and remarkably deshielded singlet signal at δ_H 12.4 ppm. Three of the four sp^2 protons constituted isolated spin systems. The presence of a key substructural moiety corresponding to a 1,3-dimethylpentenyl unit directly bonded to a quaternary oxygenated sp^2 carbon was established by analysis of COSY and HMBC spectra (**Supporting Information**). Searching for such a substructure in the Chapman & Hall Dictionary of Natural Products Database (v25.1) revealed its presence in laccaridiones A (**2**) and B (**3**) [6] and

(1) R = OH
(2) R = OCH₃
(3) R = OCH₂CH₃
(4) R = H

▸ **Fig. 1** Structure of laccaridione C (**1**), the related laccaridiones A (**2**) and B (**3**), and leptosphaerodione (**4**).

leptosphaerodione (**4**) [7]. The closely related molecular formulae, UV and NMR spectra reported for these compounds unequivocally indicated that **1** belonged to the same structural class. The UV (DAD) spectrum of **1** (**Supporting Information**) displayed the characteristic bands of the *ortho*-benzoquinone ring system (λ_{max} 308 and 490 nm) responsible for the strong red coloration of the compound. Further analysis of all key ^1H-^1H and ^1H-^{13}C long-range correlations observed in the COSY and HMBC spectra of **1** (▸ **Fig. 3**) allowed for establishing its full structure, which turned out to be identical to that of reported laccaridiones A (**2**) and B (**3**), but lacking the alkyl acetalic substituent and thus being a hemiacetal. The *E* stereochemistry of the double bond in the side chain was likewise established based on comparison of chemical shifts. Since **1** contains a chiral center at position C-3'of the fixed configuration, the two possible configurations at the hemiacetal carbon (two epimers at C-11) render two possible diastereomers with almost identical chemical shifts. The presence of a hemiacetal explains the splitting observed in the ^1H NMR spectrum for some signals of the lateral side chain. Two epimers at the chiral center at position 3' in an equimolar ratio were present in compound **1**, as indicated by the intensity of the split NMR signals. This is not surprising considering that formally the hemiacetal is the product of the intramolecular nucleophilic addition of an enolic hydroxy group at position C-9 over an aldehyde at position C-11. Since such an attack may take place over both faces of the carbonyl with equal probability, two epimeric hemiacetals in an equimolar ratio are formed. Thus, **1** was isolated as a mixture of diastereomers, and for this reason, its optical rotation was not measured.

The presence in the fungal extract of the related polyketides laccaridione A (**2**), laccaridione B (**3**), and leptosphaerodione (**4**), detected as trace components in the enriched fractions after LC-DAD-HRMS-based dereplication, indicates their obvious biosynthetic relationship.

Compound **1** was tested against the melanoma (A2058) cell line used for its bioassay-guided isolation, exhibiting an IC_{50} of $13.2 \pm 2.9\,\mu M$. It was also further tested against an extended panel of six human cell lines, exhibiting potent activity after MTT assays against the breast cancer MCF7 cell line with an IC_{50} value of $3.7 \pm 1.5\,\mu M$, neuroblastoma (SH-SY5Y) with an IC_{50} of $8.8 \pm 3.0\,\mu M$, liver carcinoma (Hep G2) with an IC_{50} of $11.6 \pm 4.2\,\mu M$, pancreas carcinoma (MIA PaCa-2) with an IC_{50} of $18.7 \pm 2.3\,\mu M$, lung carcinoma (A549) with an IC_{50} of $29.0 \pm 0.7\,\mu M$, and skin fibroblasts (CCD-25Sk, derived from normal skin of a patient that died from a high-grade glioma) with an IC_{50} of $20.5 \pm 1.7\,\mu M$. Doxorubicin gave IC_{50} values of 1.79 ± 0.06 (A2058), 6.99 ± 0.07 (MCF7), 0.69 ± 0.07 (SH-SY5Y), 1.95 ± 0.09 (Hep G2), 2.97 ± 0.73 (MIA PaCa-2), 10.51 ± 0.28 (A549), and $0.86 \pm 0.05\,\mu M$ (CCD-25Sk) when tested as a positive control under the same conditions. Methyl methanesulfonate (MMS) was used as a second positive control at a concentration of $4\,\mu M$, which caused a 100 % inhibition of all cell lines tested.

In conclusion, we report herein the isolation and cytotoxic properties of a new bioactive polyketide, laccaridione C (**1**), from *Montagnula* sp., an ascomycete from the Pleosporales order. The interest in the bioactivity of laccaridione derivatives can be perceived by the previously published patents concerning their use as antitumor, antifungal, antibacterial, and anti-HIV agents [9–11]. A further literature review confirmed these properties and indicates that
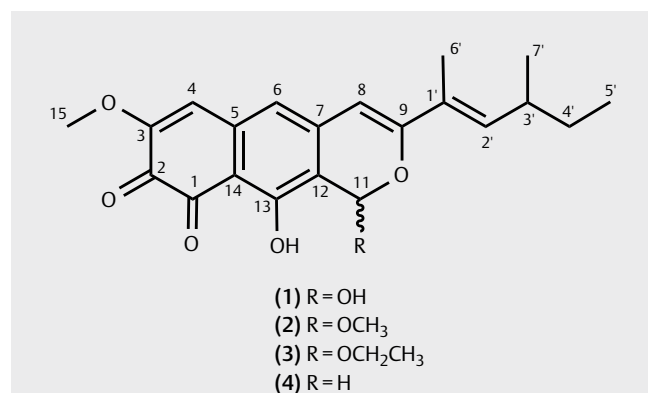
e58

Almeida C et al. Laccaridione C, a Bioactive … Planta Med Int Open 2019; 6: e57–e62
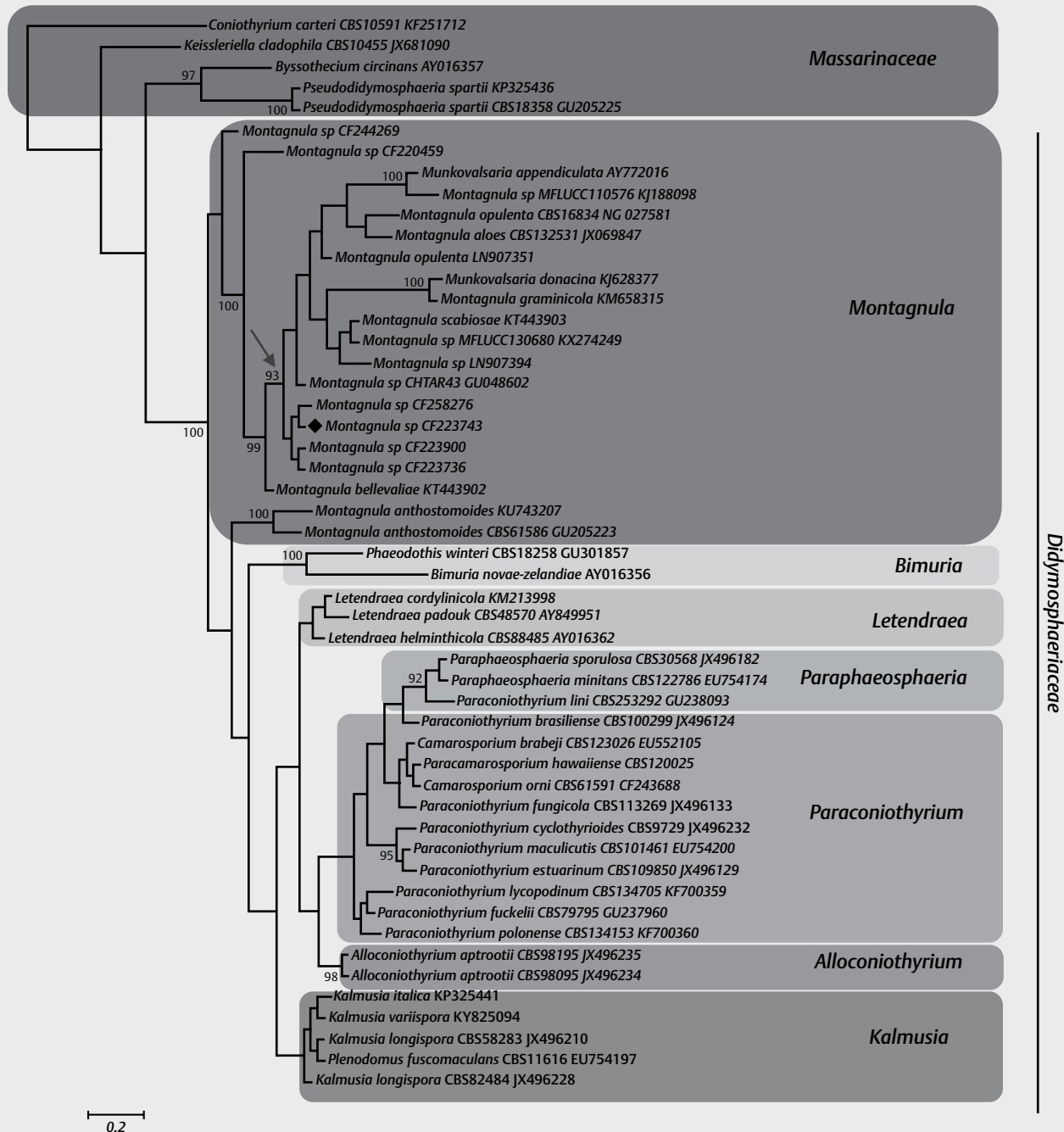
▶ **Fig. 2** Consensus tree from Bayesian-phylogeny inferences based on the 28S sequences of selected *Montagnula* strains and related genera of *Montagnulaceae*. Clade probability values are indicated at the branches. *Massarinaceae* strains were used as an outgroup.

these bioactive pigmented polyketides also have potent protease inhibitory properties [6, 12–15], which is most likely the main mechanism of action of the bioactivity herein reported for **1**.

Biosynthetically, compound **1** is the intermediate stage between the C-11 dehydroxylated form of leptosphaerodione (**4**) and the C-11 methoxylated laccaridione A (**2**) or the C-11 ethoxylated laccaridione B (**3**).

Laccaridiones and related naphtoquinones are bioactive fungal natural products that are interestingly reported from distant taxonomic groups and from marine/terrestrial environments, e. g., leptosphaerodione was first isolated from the marine-derived fungus *Leptosphaeria oraemaris*, an ascomycete of the order Pleosporales [7], and laccaridiones A and B were first isolated from the taxonomically distant terrestrial basidiomycete *Laccaria amethystea*[6]. Obionins A and B, two structurally related molecules also having

▶ **Table 1** NMR data (500 MHz, DMSO-d_6, 24 °C) for compound ▶ **1**.

No.	1	
	δ_C (ppm)	δ_H, mult. (*J* in Hz)
1	179.3	
2	175.9	
3	151.7	
4	113.3	6.67, s
5	135.6	
6	118.2	6.78, s
7	141.1	
8	99.6	6.21, s
9	156.4	
10	-	
11	87.2	6.49, d (6.1)
12	113.8	
13	162.0	
14	110.7	
15	55.5	3.75, s
1'	127.7	
2'	139.2	6.21, m
3'	34.1	2.49, m
4'	29.7	1.41, m; 1.31, m
5'	11.8	0.84, t (7.4)
6'	12.7	1.89, s
7'	20.2	0.99, d (6.6)
11-OH		7.43, d (6.1)
13-OH		12.41, s

an *ortho*-benzoquinone ring system, were isolated from the marine-derived fungus *Leptosphaeria obiones* of the Pleosporales order [16] and from an unidentified fungus also belonging to the Pleosporales order [15], respectively. New obionin derivatives were also obtained from the Sooty Blotch fungus *Microcyclospora malicola*, an ascomycete of the order Capnodiales [13]. Although rare, the presence of this natural product family has been reported in terrestrial and marine fungal strains, Basidiomycetes and in two different orders of Ascomycetes, which raises the possibility of horizontal gene transference between these fungi. The strain CF-223743 *Montagnula* sp. used in this study was isolated from the Grand Comoros Island in the Indic Ocean, raising the question if a putative intra-kingdom horizontal gene transference occurred from a microbial marine or terrestrial source. Phylogenetic placement suggests that this strain might be a new species of the *Montagnula* genus (▶ **Fig. 2**).

In summary, we report herein the isolation of laccaridione C (**1**) from the fungus *Montagnula* sp., selected as an active compound against melanoma after a microbial extract screening that increases the structural variety of this interesting bioactive chemical class, and adds a new strain to the fungal taxonomic group known to produce natural products from this polyketide heterocyclic family.

Materials and Methods

General experimental procedures

IR spectra were measured with a JASCO FT/IR-4100 spectrometer equipped with a PIKE MIRacle single reflection ATR accessory. NMR spectra were recorded on a Bruker Avance III spectrometer (500 and 125 MHz for ^{1}H and ^{13}C NMR, respectively) equipped with a 1.7 mm TCI MicroCryoProbe using the signal of the residual solvent as the internal reference (δ_H 2.50 and δ_C 39.5 ppm for DMSO-d_6). LC-UV-MS analysis was performed on an Agilent 1100 single quadrupole LC-MS system using a Zorbax SB-C8 column (2.1 × 30 mm, 5 µm, flow rate 0.3 mL/min, 40 °C). HRESIMS spectra was acquired using a Bruker maXis QTOF mass spectrometer coupled to the same HPLC system as described above [17]. Flash chromatography was performed with a CombiFlash Teledyne ISCO Rf400x. Semipreparative HPLC was done using a GILSON GX-281 322H2 coupled to a UV-VIS detector and an automatic fraction collector. Methyl ethyl ketone used for extraction was of analytical grade. All solvents employed for isolation were of HPLC grade.

Strain and fermentation

The producing fungus *Montagnula* sp. (CF-223743) was isolated from dung collected from a forest in Zikaledjou (Grand Comoros Island). The biological material was pretreated with hot water at 65 °C (50 mL/g of dung) to favor the growth of hyperthermophilic fungi. The mixture was vortexed for 5 min and the supernatant was plated in Bandoni's sorbose yeast extract tetracycline agar medium [18]. Frozen stock cultures in 10 % glycerol (− 80 °C) are maintained in the collection of Fundación MEDINA.

DNA extraction, PCR amplification, and DNA sequencing were performed as previously described [19]. The sequences of the 28S rDNA region were compared with GenBank or the NITE Biological Resource Center (http://www.nbrc.nite.go.jp/) databases using BLAST. Species and genus groups were tested with Bayesian analysis employing the Markov chain Monte Carlo approach using MrBayes 3.01 (http://mrbayes.sourceforge.net/) [20].

Ten mycelia agar plugs of the fungus were used to inoculate 50 mL of SMYA seed medium (maltose 40 g/L, yeast extract 10 g/L, neopeptone 10 g/L, agar 4 g/L) that was fermented for 7 days. This seed culture was used to inoculate (3 % v/v) 1 L of YES medium (10 × 100 mL in 500 mL Erlenmeyer) that contained Bacto yeast extract (20 g/L; Difco), $MgSO_4 \cdot 7H_2O$ (0.5 g/L; Merck), sucrose (150 g/L; Fisher), and 1 mL trace elements (from a stock of $ZnSO_4 \cdot 7H_2O$, PANREAC, 1 g/L and $CuSO_4 \cdot 5H_2O$, 0.5 g/L; Merck). All cultivations were performed at 22 °C with 70 % humidity, in agitation at 220 rpm for 14 days in Kuhner incubators.

Extraction and isolation

A 1-L culture of the fungus in YES medium was extracted with 1 L of ethyl methyl ketone (MEK). After adding the organic solvent, the mixture was subjected to continuous shaking at 220 rpm for 1 h. The biomass was separated by centrifugation, and the aqueous phase was decanted from the organic phase. The organic phase was dried to generate an extract that was loaded on a column packed with SP207SS reversed-phase resin (brominated styrenic polymer,

e60

Almeida C et al. Laccaridione C, a Bioactive … Planta Med Int Open 2019; 6: e57–e62
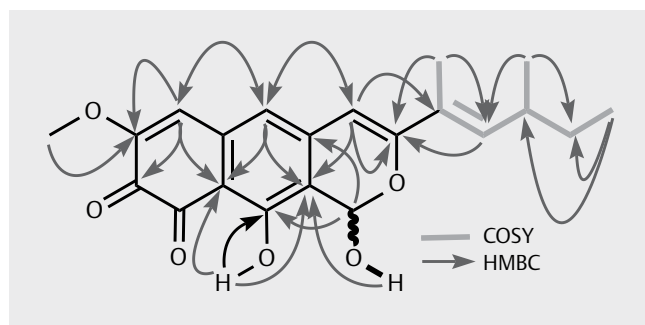
► Fig. 3 Key COSY and HMBC correlations observed in the structure of **1**.

65 g, 32 × 100 mm) previously equilibrated with water. The column was washed with water (1 L) and afterwards the sample was dissolved in DMSO, loaded onto the column, and eluted at a flow rate of 8 mL/min using a gradient from 10–100 % acetone in water (for 30 min) with a final 100 % acetone step (15 min), collecting 19 fractions of 20 mL. Fractions were concentrated to dryness on a centrifugal evaporator and activity against the melanoma (A2058) cell line was found in fractions 10 and 11. Fraction 10, selected for further purification, was dissolved in 700 µL of DMSO and subjected to repeated injections (100 µL/injection) in reversed-phase semipreparative HPLC (Agilent Zorbax RX-C8, 9.4 × 250 mm, 5 µM, 3.6 mL/min, UV detection at 210 nm) with a double isocratic solvent system of 40/60 acetonitrile/water for the first 20 min, followed by a 45/60 acetonitrile/water solvent system between minutes 20.5 and 46 to yield compound **1** (1.6 mg, retention time 43 min, 95.1 % purity by HPLC-UV at 210 nm) as responsible for the observed bioactivity.

(*E*)-1,10-Dihydroxy-7-methoxy-3-(4-methylhex-2-en-2-yl)-1H-benzo[*g*]isochromene-8,9-dione (Laccaridione C) (**1**): Dark reddish amorphous solid; UV λ_{max} (nm): 242, 308, and 490 nm; IR (ATR) v_{max} 2956, 2915, 2850, 1736, 1682, 1595, 1546 cm^{-1}; for ^{1}H and ^{13}C NMR data see **► Table 1**. HRESIMS *m/z* 371.1489 [M + H]$^+$ (calcd. for $C_{21}H_{23}O_6{}^+$, 371.1479).

Cytotoxicity MTT assays

MTT is a colorimetric assay for measuring the activity of cellular enzymes that reduce the tetrazolium dye, MTT, to purple colored formazan crystals. This assay measures mitochondrial metabolic activity via NAD(P)H-dependent cellular oxidoreductase enzymes and may, under defined conditions, reflect the number of viable cells present [21]. Six human tumor cell lines, including A2058 (ATCC CRL-11147), MCF7 (ATCC HTB-22), SH-SY5Y (ATCC CRL-2266), Hep G2 (ATCC HB-8065), MIA PaCa-2 (ATCC CRM-CRL-1420), and A549 (ATCC CCL-185), and skin fibroblast cell line CCD-25Sk (ATCC CRL-1474) were used for this study. Cells were seeded at a concentration of 1 × 10^4 cells/well in 100 µL culture medium and incubated at 37 °C in 5 % CO$_2$. After 24 h, when the monolayer was formed, the medium was replaced with a final volume of 195 µL and 5 µL of extracts and controls (extract screening) or 199 µL and 1 µL of pure compounds and controls (pure compound screening, a 10-point curve starting at a concentration of 50 µM with serial ½ dilutions). Methyl methanesulfonate (MMS) (purity 99.8 % by GC;

Sigma-Aldrich) and DMSO were used as a positive and negative controls, respectively. Doxorubicin (purity ≥ 98 % by HPLC; Sigma-Aldrich) was also tested using an 8-point curve with an initial concentration of 50 µM and serial ½ dilutions. After the extracts/compounds and controls were added, the plates were incubated at 37 °C in 5 % CO$_2$ for 72 h. After that time, the MTT solution was prepared at 5 mg/mL in PBS 1× and then diluted to 0.5 mg/mL in MEM without phenol red. The sample solution in wells was tossed, the wells were washed lightly with PBS 1×, and 100 µL of MTT dye were added to each well. The plates were then incubated for 3 h at 37 °C in a 5 % CO$_2$ atmosphere. The supernatant was removed and 100 µL of DMSO 100 % were added. The plates were gently shaken to solubilize the formed formazan crystals and the absorbance was measured using a Wallac 1420 VICTOR microplate reader at a wavelength of 570 nm. The resulting data were analyzed with Genedata Screener 12.0.5 Standard Software.

Supporting information

NMR spectra of compound **1** (**► Figs. 1S–5S**), HRMS spectrum of **1** (**► Fig. 6S**), UV spectrum of **1** (**► Fig. 7S**), images of cultures of *Montagnula* sp. in several solid media (**► Fig. 8S**), and growth inhibition curves for cytotoxic activity of compound **1** (**► Figs. 9S–15S**) are available as Supporting Information (**► Fig. 16S**)

Acknowledgements

Conflict of Interest

The authors declare no conflict of interest.

References

[1] Mondal S, Bandyopadhyay S, Ghosh MK, Mukhopadhyay S, Roy S, Mandal C. Natural products: Promising resources for cancer drug discovery. Anticancer Agents Med Chem 2012; 12: 49–75

[2] Demain AL, Sanchez S. Microbial drug discovery: 80 years of progress. J Antibiot 2009; 62: 5–16

[3] McCourt C, Dolan O, Gormley G. Malignant melanoma: A pictorial review. Ulster Med J 2014; 83: 103–110

[4] Morton DL, Essner R, Kirkwood JM, Wollman RC. Malignant melanoma. In: Kufe DW, Pollock RE, Weichselbaum RR, Bast RC, Gansler TS, Holland JF, Frei E, Editors Holland-Frei Cancer Medicine. 6th ed. Hamilton: BC Decker Inc; 2006: 1644–1662

[5] Al Qathama A, Prieto JM. Natural products with therapeutic potential in melanoma metastasis. Nat Prod Rep 2015; 32: 1170–1182

[6] Berg A, Reiber K, Dörfelt H, Walter G, Schlegel B, Gräfe U. Laccaridiones A and B, new protease inhibitors from *Laccaria amethystea*. J Antibiot 2000; 53: 1313–1316

Almeida C et al. Laccaridione C, a Bioactive … Planta Med Int Open 2019; 6: e57–e62

e61

[7] Guerriero A, D'Ambrosio M, Cuomo V, Pietra F. A novel, degraded polyketidic lactone, leptosphaerolide, and its likely diketone precursor, leptosphaerodione. Isolation from cultures of the marine ascomycete *Leptosphaeria oraemaris* (Linder). Helv Chim Acta 1991; 74: 1445–1450

[8] Perez-Victoria I, Martin J, Reyes F. Combined LC/UV/MS and NMR Strategies for the Dereplication of Marine Natural Products. Planta Med 2016; 82: 857–871

[9] Berg A, Gaefe U, Reiber K, Dahse HM, Doerfelt H. New laccaridione compounds useful as protease inhibitors, e. g. for treating cancer, autoimmune diseases and allergies. DE Patent 10037982 A1: 2002

[10] Berg A, Würzner R, Doerfelt H. Using fungal naphthopyran-8,9-diones as inhibitors of aspartyl protease, useful for treatment of infection by *Candida* or human immune deficiency virus. DE Patent 10349511 A1: 2005

[11] Tamio M, Tetsuya T, Katsuhiko A, Shingo K. EC1007 Compound. JP Patent 2000053674 2000

[12] Boss D, Maurhofer M, Schläpfer E, Défago G. Elsinochrome A production by the bindweed biocontrol fungus *Stagonospora convolvuli* LA39 does not pose a risk to the environment or the consumer of treated crops. Plant Pathol 2007; 59: 194–205

[13] Surup F, Medjedovic A, Schroers HJ, Stadler M. Production of Obionin A and Derivatives by the Sooty Blotch Fungus *Microcyclospora malicola*. Planta Med 2015; 81: 1339–1344

[14] Falkensammer B, Pleyer L, Ressler S, Berg A, Borg-von Zepelin M, Nagl M, Lass-Flörl C, Speth C, Dierich MP, Würzner R. Basidiomycete metabolites attenuate virulence properties of *Candida albicans in vitro*. Mycoses 2008; 51: 505–514

[15] Ayers S, Graf TN, Adcock AF, Kroll DJ, Shen Q, Swanson SM, Wani MC, Darveaux BA, Pearce CJ, Oberlies NH. Obionin B: An o-pyranonaphthoquinone decaketide from an unidentified fungus (MSX 63619) from the Order Pleosporales. Tetrahedron Lett 2011; 52: 5128–5230

[16] Poch GK, Gloer JB. Obionin A: A new polyketide metabolite from the marine fungus *Leptosphaeria obiones*. Tetrahedron Lett 1989; 30: 3483–3486

[17] Martín J, Crespo G, González-Menéndez V, Pérez-Moreno G, Sánchez-Carrasco P, Pérez-Victoria I, Ruiz-Pérez LM, González Pacanowska D, Vicente F, Genilloud O, Bills GF, Reyes F. MDN-0104, an antiplasmodial betaine lipid from *Heterospora chenopodii*. J Nat Prod 2014; 77: 2118–2123

[18] Bills GF, Foster MS. Appendix II. Formulae for selected materials used to isolate and study fungi and fungal allies. In: Mueller GM, Bills GF, Foster MS, Editors Biodiversity of Fungi: Inventory and Monitoring Methods. Boston: Elsevier Academic Press; 2004: 595–618

[19] Gonzalez-Menendez V, Martin J, Siles JA, Gonzalez-Tejero MR, Reyes F, Platas F, Tormo JR, Genilloud O. Biodiversity and chemotaxonomy of *Preussia* isolates from the Iberian Peninsula. Mycol Progress 2017; 16: 713–728

[20] Ronquist F, Huelsenbeck JP. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 2003; 19: 1572–1574

[21] Patel MR, Patel SK. Cytotoxic activity of methanolic extract of *Artocarpus heterophyllus* against A549, Hela and MCF-7 cell lines. J Appl Pharm Sci 2011; 1: 167–171