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The antimicrobial potential of algicolous marine fungi for counteracting multidrug resistant bacteria: phylogenetic diversity and chemical profiling

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e cmonti@unisa.it
Abstract

Marine fungi represent an important but still largely unexplored source of novel and potentially bioactive secondary metabolites. The antimicrobial activity of nine sterile mycelia isolated from the green alga *Flabellia petiolata* collected from the Mediterranean Sea was tested on four antibiotic resistant bacterial strains using extracellular and intracellular extracts obtained from each fungal strain. The isolated fungi were identified at the molecular level and assigned to one of the Dothideomycetes, Sordariomycetes or Eurotiomycetes classes. Following assessment of the inhibition of bacterial growth (IC$_{50}$), all crude extracts were subjected to preliminary $^1$H NMR and TLC analysis. According to the preliminary pharmacologic, spectroscopic/chromatographic results, extracts of the fungal strains MUT 4865, classified as *Beauveria bassiana*, and MUT 4861, classified as Microascacea sp.2, were selected for LC-HRMS analysis. Chemical profiling of antibacterial extracts from MUT 4861 and MUT 4865 by LC HRMS allowed the identification of the main components of the crude extracts. Several sphingosine bases were identified, including a compound previously unreported from natural sources, which gave a rationale to the broad spectrum of antibacterial activity exhibited.

Keywords

Antimicrobial compounds; bioactive fungal compounds; marine fungi; marine natural products; multidrug resistant bacteria; sphingosine bases.
Introduction

The worldwide diffusion of antibiotic-resistant microorganisms requires the development of new, efficient antimicrobial molecules. For more than half a century, the main strategy for obtaining new antimicrobial agents has consisted of semisynthetic remodeling of natural products. However, drugs obtained in this way are only temporarily effective against pathogenic microorganisms, which develop antibiotic resistance [1]. The problem regarding microbial resistance to antibiotics may be overcome by the discovery of new natural products, which, due to their chemical novelty, could inhibit unknown single or multiple microbial targets.

The search for natural products of pharmaceutical interest in the marine environment has been progressing at an unprecedented rate, resulting in the discovery of a number of molecules, many of which have new carbon skeletons and interesting biological activities [2, 3].

Among marine microorganisms, fungi play a crucial role, being a reservoir of biologically active secondary metabolites [4-6]. Recently, several new metabolites from marine fungi have been reported to display notable antibacterial activities [7-9]. Despite their proven biosynthetic potential, scientific research has not intensively focused on marine fungi for seeking new drugs [10]. However, promising fungi are equipped with gene clusters potentially involved in the biosynthesis of secondary metabolites [11]. Therefore, research into the isolation, identification and characterization of new fungal strains, capable of producing useful bioactive natural compounds, should be carried out.

Hence, the aim of this work was to assess the antibacterial potential of nine sterile mycelia isolated from the green alga Flabellia petiolata collected from the Mediterranean Sea, against some representative multidrug resistant (MDR) bacteria, relevant in Cystic Fibrosis and nosocomial infections, and to analyze the chemical profiles of the most active fungal crude extracts.
Materials and Methods

Fungal strains

Fungi were isolated and roughly identified from the green alga *F. petiolata* collected in March 2010 near to Elba Island in the Mediterranean Sea [12], and are preserved at the Mycotheca Universitatis Taurinensis - MUT (DBIOS - University of Turin). All the selected fungi were revealed to be sterile mycelia and were identified by molecular analysis (Table 1).

Molecular, Bioinformatics and Phylogenetic analyses

Genomic DNA was extracted using Cetyl Trimethyl Ammonium Bromide (CTAB, Sigma-Aldrich St. Louis, USA) according to the protocol of Graham et al. [13].

The nrDNA Internal Transcribed Spacer (ITS) and Large ribosomal SubUnit (LSU) partial regions were amplified using the universal primers ITS1F/ITS4 (Sigma-Aldrich St. Louis, USA) and LR0R/LR7, as previously described [14].

Amplification products were sequenced at Macrogen Europe (The Netherlands). Sequences were checked and assembled using Sequencher 4.9 software and compared to those available in the GenBank database using the BLASTn option of the BLAST program (www.blast.ncbi.nlm.nih.gov) and CBS Mycobank Pairwise Sequence Alignment (www.mycobank.org). Newly generated sequences were deposited in the GenBank database and were assigned the accession numbers reported in Table 1.

Phylogenetic analysis was only performed on LSU sequences, as comparable ITS sequences of fungi studied in this article are rarely found in public databases and/or poorly informative. LSU sequences were selected for phylogenetic analysis on the basis of BLASTn and CBS results. Two sequences datasets were composed, following reference [14] for Pleosporales and reference [15] for Sordariomycetes.

Alignments were generated using MEGA 5.10 [16] and manually refined. Phylogenetic analyses were performed using both Bayesian Inference (BI; MrBayse3.2.2)
and Maximum Likelihood (ML; RAxML v.7.3.2) [18] approaches, as previously described [14]. Bayesian Posterior Probability (BPP) values over 0.6 (with MLB over 50%) are reported in the resulting trees.

**Fungal growth conditions**

Preliminary growth condition tests were performed in order to define the most effective and appropriate medium to induce the production of bioactive secondary metabolites in the selected fungal strains. Each fungal strain was inoculated in duplicate by 10 agar plugs of 5 mm diameter cut from the edge of actively growing culture onto malt extract agar in 150 ml flasks containing 100 ml of three different media: PCB (10 g of crushed potatoes and 10 g of crushed carrots in 1 L of ddH$_2$O), MeCl (20 g malt extract, 17 g NaCl in 1 L of ddH$_2$O) and WST30 (10 g glucose monohydrate, 5 g soya peptone, 3 g malt extract, 3 g yeast extract, 30 g NaCl). Flasks were incubated in the dark at 24°C and rotated at 150 rpm. The broth and mycelium of each strain were collected after 2 and 4 weeks and submitted to an extraction procedure for the preliminary bio-chemical analysis (see below). The MeCl medium and 4 week-incubation were selected as the best conditions (24°C in the dark). Hence, each fungus was inoculated (100 agar plugs of 5 mm diameter) in 2 L flasks containing 1.5 L of MeCl, which was incubated in the dark at 24°C, at 180 rpm for 4 weeks.

**Extract preparation**

Samples were centrifuged at 11,200 x rcf for 30 min at 4 °C and filtrated in order to separate the mycelium from the culture broth. Supernatants were extracted with ethyl acetate (EtOAc) and the resulting extracts were dried-out by using a Rotavapor, weighed, solubilized in dimethyl sulfoxide (DMSO, 100%) at a final concentration of 100 mg/mL and stored at -20°C. The presence of antimicrobial compounds in the mycelia was also evaluated. In order to efficiently lyse the cells, different mechanical disruption methods were used in a sequential
manner. The first step consisted of homogenization with Ultra Turrax T25 (IKA-Werke, Staufen, Germany). The homogenate was then washed twice with 20 mL of EtOAc to recover the intracellular extract; in addition, to improve the fungal lysis, mycelia were treated with liquid nitrogen (15 mL N₂/g mycelium). Samples were transferred into a pre-cooled mortar and minced under liquid nitrogen with a pestle and washed twice with 20 mL of EtOAc. At the last step, to completely destroy the membrane, all the mycelium was transferred and processed in a Potter-Elvehjem homogenizer (Sigma-Aldrich, Saint Louis, MO) in the presence of EtOAc. Subsequently, the powdered mycelium was transferred into a separator funnel and mixed five times with two volumes of EtOAc. In order to increase the yield of some extracts, mycelia were further soaked in acetone for 18 hours under agitation. The whole EtOAc and acetone fractions were collected and dried-out by using a Rotavapor. Final extracts were weighed, solubilized in DMSO (100%) at a final concentration of 100 mg/mL and stored at -20°C.

**Antimicrobial assay**

The extracts produced as such were checked for the ability to inhibit the growth of a selected panel of human pathogens. An IC₅₀ assay was used to evaluate the concentration of the extracts at which bacterial target growth was inhibited by 50%. The following multidrug resistant bacteria were used for the antimicrobial screening: *Burkholderia metallica* LMG 24068 [19], *Pseudomonas aeruginosa* PA01 [20], *Klebsiella pneumoniae* DF12SA [21] and *Staphylococcus aureus* 6538P [22]. All bacteria were routinely grown at 37°C in Lysogeny broth (5 g yeast extract, 10 g sodium chloride, 10 g tryptone in 1 L of ddH₂O), with the exception of *S. aureus*, which was grown in Mueller Hinton Broth (Applichem, Darmstadt, Germany).

Extracts were placed into each well of a 96-well microtiter plate at an initial concentration of 2 mg/mL and serially 2-fold diluted using the appropriate medium. Wells
containing only DMSO (2% v/v) were used as a control to determine the effect of this solvent on bacterial growth.

Cells were prepared as follows: a single colony of each pathogenic strain was used to inoculate 3 mL of liquid medium in a sterile bacteriological tube. After 5-8 h of incubation, growth was measured by monitoring the absorbance at 600 nm and about 40,000 colony-forming units were dispensed into each well of the prepared plate. Plates were incubated at 37°C for 20 h and growth was measured using a VICTOR X Multilabel Plate Reader (PerkinElmer, Waltham, MA) by monitoring the absorbance at 600 nm.

**Metabolic profiling of crude extracts**

All crude extracts were subjected to Thin Layer Chromatography (TLC) analysis and 

$^1$H Nuclear Magnetic Resonance (NMR). TLC analysis was carried out on Alugram silica gel G/UV254 plates with solvent mixture of different polarity using vanillin reagent as revelation system; $^1$H NMR analysis were performed with Varian INOVA 400 MHz instrument, in CDCl$_3$ solvent, at room temperature with tetramethyilsilane (TMS) as internal reference.

Selected extracts were analyzed using a LTQ XL Liquid Chromatography-High Resolution Mass Spectrometry system (LC-HRMS) (ThermoScientific) equipped with the Accelera 600 Pump and Accelera Auto Sampler system. A volume of 10 µl of sample was injected at a concentration of 10 mg/mL in methanol. The mixture was separated on a Phenomenex LUNA C8 (150 X 2.1 mm, 5 µm particle size) column at a flow rate of 200 µL/min, using an acetonitrile-water gradient. Mobile phase A was 90% H$_2$O 10% acetonitrile (ACN) 0.1% formic acid (FA) and mobile phase B was 10% H$_2$O 90% ACN 0.1% FA; the gradient started at 10% B up to 90% B in 70 min, was kept at 90% of B for 10 min before the re-equilibration step. The mass spectrometer operated in positive electrospray ionization (ESI) mode, at 4 kV capillary voltage and 280°C. The calibration procedure was carried out using ThermoScientific positive calibration solution composed of caffeine, MRFA and Ultramark.
All spectra were acquired in the m/z range from 280 to 700 u.m.a., setting resolution at 30,000; MSMS spectra were acquired in an opportune m/z range using 35 of collision energy. Thermo Scientific software Xcalibur was used to obtain molecular formulas. The Molecular Formulas (MF) deduced by High-Resolution Electrospray Ionization Mass Spectrometry (HRESIMS) were checked by available data banks [23-25] and, in the case of alternative structures, they were discriminated by MS^n analysis using the data available in the literature [26] or ex-novo analysis, and then by checking diagnostic signals in the ^1H NMR spectrum of the crude extracts.

Results

Phylogeny and taxonomic identification of the fungal isolates

The molecular and phylogenetic analysis revealed that strains MUT 4859, MUT 4860, MUT 4883, MUT 4886, and MUT 4966 belong to the order Pleosporales (Dothideomycetes class). In particular, MUT 4860 was identified as Massarina sp. and MUT 4883 as Biatriospora sp., both clustering in the Biatriosphoraceae family, while MUT 4859, MUT 4886 and MUT 4966 were identified at the family level (Roussoellaceae, Supplementary materials Fig. S1) [27].

MUT 4861, MUT 4865, and MUT 4885 belonged to the Sordariomycetes class; specifically, MUT 4865 belonged to Beauveria bassiana, while MUT 4861 and MUT 4885 clustered within the Microascaceae family (Supplementary materials Fig. S2).

Finally, MUT 4979 was identified as Knufia petricola (syn. Sarcinomyces petricola, Incertae sedis, Chaetothyriales, Eurotiomycetes) by both ITS and LSU sequences (homology percentage = 99%).

Antimicrobial activity

In order to select the best growth medium for producing the antimicrobial compounds, preliminary extractions and antimicrobial assays were performed on small-scale cultures of
fungi grown in MeCl, PCB and WST30. These analyses demonstrated that fungi grown in MeCl exhibited the highest degree of antimicrobial activity (Supplementary materials Table S1). This medium was therefore selected for further experiments. Moreover, the antimicrobial potentials of the extracellular and intracellular extracts were compared; results revealed that the latter exhibited the highest yield and activity (Supplementary materials Table S2).

Starting from these preliminary results, extracts obtained from mycelium lysates were used for the antimicrobial screening, targeting a panel of MDR human pathogens. The antimicrobial activity displayed by the different fungal strains against the four MDR bacteria is reported in Table 2 as IC\textsubscript{50} values. The resistance of each strain to Ampicillin, Chloramphenicol, Kanamycin, Tetracycline and Trimethoprim was confirmed and IC\textsubscript{50} values are reported in Table S3 (Supplementary materials).

Extracts produced from strains MUT 4861, MUT 4865, and MUT 4979 resulted as being the most active and promising ones. In particular, MUT 4861 was able to strongly inhibit \textit{B. metallica} (IC\textsubscript{50} 0.5-0.25 mg/mL) and \textit{S. aureus}, and was the only one to show, by both EtOAc and acetone extracts, an inhibitory effect against \textit{P. aeruginosa}. Both extracts from MUT 4865 were able to inhibit \textit{B. metallica} and \textit{S. aureus} (IC\textsubscript{50} 0.5-0.25) and the EtOAc extracts also showed inhibition against \textit{K. pneumoniae}. No effects were observed against \textit{P. aeruginosa}. The extract from MUT 4979 showed antimicrobial activity against three out of the four pathogens (IC\textsubscript{50} 1.0-0.25), with the exception of \textit{K. pneumoniae}. Extracts of MUT 4859, 4860, and 4966 only showed a significant activity against \textit{B. metallica} and \textit{S. aureus}, which were the most sensitive bacterial strains to the fungal extracts. MUT 4883, 4885 and 4886 extracts were the weakest strains showing no significant effects against the target bacteria. Acetone extracts showed similar antimicrobial activity compared to EtOAc extracts. The only exception was MUT 4861, of which the acetone extract was more active than the EtOAc extract.
Overall, the most promising strains were MUT 4865, 4979 and 4861, which exhibited the highest degree of antibacterial activity.

**Secondary Metabolite Analyses**

Based on the results of the preliminary pharmacologic, spectroscopic and chromatographic screening, the extracts of MUT 4865 and MUT 4861 were selected for the chemical profiling and were analyzed by LC-HRMS. Other strains did not produce detectable amounts of secondary metabolites under cultivation conditions and, therefore, revealing their potential of secondary metabolite production will require further investigation.

**Beauveria bassiana** MUT 4865: both acetone and EtOAc extracts were subjected to HRESIMS analysis (Fig. 1A). Compound 1 analyzed for C$_{22}$H$_{43}$O$_2$N by HRMS analysis (calculated for C$_{22}$H$_{43}$NO$_2$Na: 376.3192, found [M + Na]$^+$: 376.3195). In the MS$^2$ spectrum (Supplementary materials Fig. S3), the sequential loss of one ammonia and two neutral water molecules indicated the presence of one amino and two hydroxyl groups. The planar structure of this compound was deduced from the analysis of the MS$^3$ spectrum, which showed a fragmentation pattern compatible with the localization of the two double bonds at the unusual positions of 6 and 17, revealing that it corresponded to the long chain sphingadienine (Fig. 2). Therefore a 1,3-dihydroxy-2-amino-6,17-docosadiene structure was tentatively proposed. Assignment of the relative configuration of the two contiguous stereogenic centers, as well as of the two double bonds would require isolation of the compound from a large-scale cultivation batch of the fungal strain.

As shown in Fig. 1B, the acetone extract did not contain a detectable amount of compound 1, whereas some sphingosine compounds were detected, such as phytosphingosine (2), dihydrosphingosine (3) and phytoceramide C2 (4). The MS$^2$ pattern analysis (Table 3 and Supplementary materials Fig. S4-S6) leads to a straightforward assignment of a planar structure to these compounds.
Compound 5, which was present in both EtOAc and acetone extracts, was tentatively identified as aphidicolin; compound 6 was tentatively identified as fusoxysporone and compound 7, a minor component of the EtOAc extract, was identified as bis (2-ethylhexyl) hexanedioic acid.

Microascacea sp.2 MUT 4861: the EtOAc extract contained a very complex mixture of lipid and polysaccharide components, evidenced by $^1$H NMR analysis, which, however, did not allow its de-replication by HRESIMS. Conversely, the main components of the acetone extract were identified. For this fungal strain, two polar components were revealed to be sphingoid bases.

In addition to phytosphingosine (2), an "unusual" sphingoid base with a molecular formula C$_{19}$H$_{39}$NO$_3$ was detected. The MS$^2$ spectrum (Supplementary materials Fig. S10) showed fragmentation peaks resulting in the sequential loss of three water molecules, whereas no ammonia elimination was measured. This finding could suggest the involvement of a nitrogen atom in an azetidine ring, as in isomeric penaresidins A and B.

Although the fragmentation pattern observed in the MS$^3$ spectrum (Supplementary materials Fig. S11) is compatible with these structures, no ambiguous information relative to the position of the hydroxyl groups, of the methyl branching, or even on the nature of unsaturation, can be drawn.

Finally, Scopularide A (8) [28] was identified by MF analysis and by diagnostic MS$^2$ fragmentations (Table 4 and Supplementary materials Fig. S9).

**Discussion**

In this study, the green marine alga *F. petiolata* was chosen as a source of promising marine fungi since it has been previously demonstrated that fungi isolated from marine algae showed strong antimicrobial activity against several human pathogenic bacteria [29], probably deriving from the ability to protect their algal host from external threats [30].
Identifying new fungal strains could lead to the discovery of new and unusual compounds, which can be utilized for biotechnological and pharmaceutical applications.

The first step of this work was the phylogenetic affiliation of fungal strains, which was carried out according to molecular and phylogenetic analysis. *Massarina* sp. (MUT 4860) and *Biatriospora* sp. (MUT 4883) clustered in the Biatriosporaceae family, which accommodate genera that have often been collected from a range of both terrestrial and aquatic hosts, and are commonly found in decaying submerged intertidal mangrove wood [27]. Recently, it has been demonstrated that a strain identified as *Biatriospora* sp. is an efficient producer of secondary metabolites, in particular naphthoquinone derivatives [31].

*MUT 4859, MUT 4886 and MUT 4966 clustered in the Roussoellaceae family, which includes species of saprobic fungi isolated from decaying bamboo culms or palm fronds [32].

*Beauveria bassiana* (MUT 4865) is a marine isolate of well-known entomopathogenic fungus, commonly isolated from decaying arthropods or from plant tissue as an endophyte [33].

On the basis of molecular and phylogenetic data, MUT 4861 and MUT 4885 could be considered as putative new species and even new genera of the Microascales, a small order of primarily saprobic fungi in soil, rotting vegetation and dung. Some species of this order are responsible for plant diseases, while other members cause human diseases [34].

*Knufia petricola* (MUT 4979) is an algicolous strain of microcolonial fungus with a meristematic-black yeast morphology, which has only been previously found on stone substrates, such as unlichenized fungus with its natural ecological niche [35]. To the best of our knowledge, this is the first report of the presence of this species in a marine environment.

As the antimicrobial activity of these algicolous fungi on MDR bacteria (according to the results of the bioassay tests) were in agreement with the known antimicrobial potential of marine fungi, further investigations are certainly recommended, also considering the value of
producing antimicrobial compounds from new taxonomic entities that have never been
previously explored.

The most promising fungal strains were MUT 4865, 4979 and 4861, which exhibited
the highest degree of antibacterial activity. MUT 4865, identified as B. bassiana,
representatives of which are well-known producers of insecticidals and antimicrobials [36],
showed a strong activity against all the pathogens tested. For K. petricola (MUT 4979), this is
the first report of an antimicrobial activity exhibited by the fungal extracts from this species.
Further studies are necessary, considering that the class this organism belongs to
(Eurotiomycetes) includes several species (e.g. Aspergillus spp., Paecilomyces spp.,
Penicillum spp.) that have been reported to be a source of many antimicrobial metabolites [37, 38].

Finally, MUT 4861 is of special interest due to the fact that it is presumed to belong to
a new species of Microascaceae, a family that includes a number of fungi capable of
producing several antimicrobial secondary metabolites [37, 38].

The chemical profiling of the most active crude extracts have highlighted the presence
of chemically diverse metabolites. In particular, both strains were found to contain sphingoid
bases. Diverse variants of the long chain bases sphingosine and phytosphingosine have been
reported from marine organisms, especially sponges and tunicates [39, 40], but to the best of
our knowledge, this is the first report of sphingosine-free bases from marine fungi.

In particular, the long chain sphingadienine 2-aminodocosa-6,17-dien-1,3-diol has
never been described as a free base or as a component of polar lipids from natural sources.
The related docosa-4,15-sphingadienine and 4-hydroxy-docosa-15-sphingenine have been
reported as components in sphingophosphonolipids from the marine gastropod Turbo
cornutus [41]. Noteworthy, recent years have witnessed an ever-increasing interest towards
the so-called “sphingoid bases” for their role in the regulation of physiological and
pathological conditions [42]. In particular, a recent study [43] revealed that sphingoid long-chain bases displayed antibacterial activity against a broad spectrum of pathogenic bacteria, including *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Haemophilus influenzae*, *Moraxella catarrhalis* and even *Burkholderia cepacia*, at nanomolar-to-low micromolar concentrations. Therefore, even though we cannot exclude, *a priori*, the possibility that the antimicrobial activity could rely on the combination of different molecules, compound 1, and co-occurring sphingosines 2, 3 and 4, previously reported as common components of fungal membrane sphingolipids [44], may be responsible for the antimicrobial effects exhibited by MUT 4865 crude extracts towards the pathogenic bacteria investigated so far. However, tests with the purified compound will be necessary to validate this hypothesis.

Regarding the other tentatively identified components of MUT 4865 extracts, aphidicolin is a tetracyclic diterpene with known antiviral and antimitotic properties, first isolated from the fungus *Cephalosporum aphidicola* [45]. Fusoxysporone, is a viscidane-type diterpene first isolated from *Fusarium oxysporum* [46], and is also found as a component of the cytotoxic extracts of a *Penicillium* strain isolated from bivalve mollusks [47]. To the best of our knowledge, no biological activities have been described for this compound, so far.

Compound 7, identified as bis (2-ethylhexyl) hexanedioic acid, is known as plasticizer [48] and described as a component of cyanobacteria, Antarctic [49] and terrestrial [50] strains of *Streptomyces*, and of a tropical plant [51].

Sphingosine-related compounds were also detected in the EtOAc extract of Microascacea sp.2 MUT 4861, which also contains a member of the class of so-called anhydrophytosphingosines, in particular the detected compound is isomeric with azetidine-derived penaresidins A and B, which were first isolated from the marine sponge *Penares* sp. [52].

Conversely, compound 8 is a cyclodepsipeptide scopularide A, a molecule with
antiproliferative activity, previously isolated from a marine strain of the fungus *Scopulariopsis brevicaulis* [28], belonging to the same Microascaceae family assigned to MUT 4861.

In conclusion, nine selected strains isolated from the green alga *F. petiolata* were chosen as a promising source of antimicrobial compounds. All fungal strains demonstrated interesting antimicrobial activity against four human pathogenic MDR bacteria. Crude extracts of three of the selected fungal strains, preserved at the MUT collection as MUT 4865, MUT 4979 and MUT 4861, were able to strongly inhibit the entire panel of pathogens. The chemical profiling of the antibacterial extracts from *B. bassiana*, MUT 4865, and Microascaceae sp.2, MUT 4861, by LC HRMS allowed identification of the main components of the crude extracts. No detectable amounts of peptide mycotoxins, such as beauvericin or enniatins, known for their antimicrobial and anti-tumor activities [53], were detected. Isolation of several sphingosine bases, including compound 1, previously unreported from natural sources, gave a rationale to the broad spectrum of antibacterial activity exhibited by the crude extract of this fungal strain. Further experiments aimed at the isolation of pure compounds and determination of their biological activity are currently underway.

**Conflicts of Interest**

The authors declare that there are no conflicts of interest.

**Acknowledgments**

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References


Legends to figures

**Fig. 1.** ESI positive mode base peak chromatograms of the active samples MUT 4865 EtOAc extract (panel A), Acetone extract (panel B) and MUT 4861 Acetone extract (panel C). Numbers above the peaks identify the metabolites listed in Tables 2 and 3.

**Fig. 2.** MS$^3$ ESI positive mode spectrum of the precursor ion at $m/z$ 359.30 derived from MSMS at $m/z$ 376.31 and its proposed fragmentation.

**Fig. 3.** Chemical structures of secondary metabolites (1-8) identified by LC-HRMS in the bioactive extracts of *Beauveria bassiana* MUT 4865 and MUT 4861.
Table 1. MUT code, taxonomic assessment of sterile mycelia isolated from *F. petiolata* and GenBank accession numbers.

<table>
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<th>MUT Code</th>
<th>Fungal taxa</th>
<th>GenBank accession number ITS and LSU</th>
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<td>4883</td>
<td><em>Biatriospora</em> sp.</td>
<td>KR014352</td>
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<td></td>
<td></td>
<td>KP671728</td>
</tr>
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<td>4865</td>
<td><em>Beauveria bassiana</em> (Bals.-Criv.) Vuill.</td>
<td>KR014380</td>
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<tr>
<td></td>
<td></td>
<td>KP671729</td>
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<td>4860</td>
<td><em>Massarina</em> sp.</td>
<td>KR014362</td>
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<tr>
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<td></td>
<td>KP671730</td>
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<tr>
<td></td>
<td></td>
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<td>4979</td>
<td><em>Knufia petricola</em> (U. Wollenzien &amp; de Hoog) Gorbushina &amp; Gueidan</td>
<td>KR014376</td>
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Table 2. Antimicrobial activity of the fungal intracellular extracts vs four bacterial strains belonging to different species. The data are reported as capacity to inhibit the microorganisms growth in more than 50% (IC$_{50}$). Growth in the presence of 2% DMSO was considered as 100% growth. ND: Not detected.

<table>
<thead>
<tr>
<th>Fungi</th>
<th>\textit{B. metallica} LMG 24068</th>
<th>\textit{P. aeruginosa} PA01</th>
<th>\textit{K. Pneumoniae} DF12SA</th>
<th>\textit{S. aureus} 6538P</th>
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<tbody>
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<td>MUT CODE</td>
<td>Ethyl acetate</td>
<td>Acetone</td>
<td>Ethyl acetate</td>
<td>Acetone</td>
</tr>
<tr>
<td>4860</td>
<td>0.5 - 0.25</td>
<td>&gt; 2.0</td>
<td>&gt; 2.0</td>
<td>&gt; 2.0</td>
</tr>
<tr>
<td>4861</td>
<td>0.5 - 0.25</td>
<td>0.5 - 0.25</td>
<td>2.0 - 1.0</td>
<td>1.0 - 0.5</td>
</tr>
<tr>
<td>4865</td>
<td>0.5 - 0.25</td>
<td>0.5 - 0.25</td>
<td>&gt; 2.0</td>
<td>&gt; 2.0</td>
</tr>
<tr>
<td>4979</td>
<td>1.0 - 0.5</td>
<td>ND</td>
<td>1.0 - 0.5</td>
<td>ND</td>
</tr>
<tr>
<td>4966</td>
<td>1.0 - 0.5</td>
<td>ND</td>
<td>&gt; 2.0</td>
<td>ND</td>
</tr>
<tr>
<td>4885</td>
<td>2.0 - 1.0</td>
<td>ND</td>
<td>&gt; 2.0</td>
<td>ND</td>
</tr>
<tr>
<td>4886</td>
<td>2.0 - 1.0</td>
<td>ND</td>
<td>&gt; 2.0</td>
<td>ND</td>
</tr>
<tr>
<td>4883</td>
<td>2.0 - 1.0</td>
<td>ND</td>
<td>2.0 - 1.0</td>
<td>ND</td>
</tr>
</tbody>
</table>
**Table 3.** Annotated peaks observed in the chromatograms of the EtOAc and Acetone extracts of *Beauveria bassiana* MUT 4865

<table>
<thead>
<tr>
<th>RT (min)</th>
<th>MS and MS/MS</th>
<th>Suggested MF</th>
<th>Proposed structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>23.20</td>
<td>376.3195 [M+Na]⁺ (Δppm: 1.049) MS² (Fig. S3): 359.29, 341.28; MS³ see Fig. 2</td>
<td>C₂₂H₄₃NO₂</td>
<td>2-aminodocosa-6,17-dien-1,3-diol (1)</td>
</tr>
<tr>
<td>28.32</td>
<td>318.30015 (Δppm: -0.379) MS² (Fig. S4): 300.29, 282.29, 265.33</td>
<td>C₁₈H₃₉NO₃</td>
<td>2-aminoocdecan-1,3,4-triol (4-hydroxsphinganine or phytosphingosine) (2)</td>
</tr>
<tr>
<td>29.11</td>
<td>302.30543 (Δppm: 0.245) MS² (Fig. S5): 284.29, 266.31, 249.26</td>
<td>C₁₈H₃₉NO₂</td>
<td>2-aminoocdecan-1,3-diol (dihydrosphingosine) (3)</td>
</tr>
<tr>
<td>30.03</td>
<td>360.31079 (Δppm: -0.126) MS² (Fig. S6): 342.31,324.32, 300.31, 264.30, 212.19</td>
<td>C₂₀H₄₁NO₄</td>
<td>N-[1,3,4-trihydroxyoctadecan-2-yl]acetamide (phytoceramide C2) (4)</td>
</tr>
<tr>
<td>45.65</td>
<td>339.25320 (Δppm: -0.876)</td>
<td>C₂₀H₃₄O₄</td>
<td>Aphidicolin (5)</td>
</tr>
<tr>
<td>54.04</td>
<td>287.23634 (Δppm: 0.584) MS² (Fig. S7): 269.23, 203.14, 175.11</td>
<td>C₂₀H₃₆O</td>
<td>Fusoxysporone (6)</td>
</tr>
<tr>
<td>60.38</td>
<td>395.3309 (Δppm: 0.145)</td>
<td>C₂₈H₄₂O</td>
<td>Ergosta-5,7,22-trien-3-β-ol (ergosterol)</td>
</tr>
<tr>
<td>62.89</td>
<td>393.3153 (Δppm: 0.401)</td>
<td>C₂₈H₄₆O</td>
<td>Ergostane derivative</td>
</tr>
<tr>
<td>66.49</td>
<td>371.31453 (Δppm: -1.056) MS² (Fig. S8): 259.01, 240.70, 146.9, 128.9, 110.99</td>
<td>C₂₂H₄₃O₄</td>
<td>Bis(2-ethylhexyl) hexanedioic acid (7)</td>
</tr>
</tbody>
</table>
Table 4. Annotated peaks observed in the chromatograms of the Acetone extract of Microascacea sp.2 MUT 4861.

<table>
<thead>
<tr>
<th>RT (min)</th>
<th>MS and MS(^n)</th>
<th>Suggested MF</th>
<th>Proposed structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>31.52</td>
<td>318.30002 ((\Delta\text{ppm} \pm 0.756))</td>
<td>C(<em>{18})H(</em>{39})NO(_3)</td>
<td>2-amino-octadecane-1,3,4-triol (4-hydroxysphigamine or phytosphingosine) (2)</td>
</tr>
<tr>
<td></td>
<td>MS(^2) (Fig. S4): 300.29, 282.29, 265.33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>34.29</td>
<td>330.30024 ((\Delta\text{ppm} \pm 0.031))</td>
<td>C(<em>{19})H(</em>{39})NO(_3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MS(^2) (Fig. S10): 312.26, 294.33, 282.32, 256.32</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>[MS(^3) (@ 294.33)] (Fig. S11): 266.33, 168.18, 154.07, 140.11, 133.01, 126.0, 111.96, 97.94</td>
<td></td>
<td></td>
</tr>
<tr>
<td>49.05</td>
<td>672.43291 ((\Delta\text{ppm} \pm 0.166))</td>
<td>C(<em>{36})H(</em>{57})N(_5)O(_7)</td>
<td>Scopularide A (8)</td>
</tr>
<tr>
<td></td>
<td>MS(^2) (Fig. S9): 654.5, 525.3, 507.2, 454.2, 436.2, 323.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>58.14</td>
<td>409.3101 ((\Delta\text{ppm} 0))</td>
<td>C(<em>{28})H(</em>{40})O(_2)</td>
<td>Ergostane derivative</td>
</tr>
<tr>
<td>59.94</td>
<td>393.3154 ((\Delta\text{ppm} 0))</td>
<td>C(<em>{28})H(</em>{40})</td>
<td>Ergostane derivative</td>
</tr>
<tr>
<td>65.6</td>
<td>395.3307 ((\Delta\text{ppm} 0))</td>
<td>C(<em>{28})H(</em>{42})O</td>
<td>Ergosterol</td>
</tr>
<tr>
<td>73.06</td>
<td>371.31576 ((\Delta\text{ppm} 0))</td>
<td>C(<em>{22})H(</em>{42})O(_4)</td>
<td>Bis(2-ethylhexyl) hexanedioic acid (7)</td>
</tr>
<tr>
<td></td>
<td>MS(^2) (Fig. S8): 259.01, 240.70, 146.9, 128.9, 110.99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>77.20</td>
<td>377.32019 ((\Delta\text{ppm} 0))</td>
<td>C(<em>{28})H(</em>{40})</td>
<td>Ergosta-3,5,7,9(11),22-pentaene</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Supporting Information

The antimicrobial potential of algicolous marine fungi for counteracting multidrug resistant bacteria: phylogenetic diversity and chemical profiling

Giorgio Gnavi, Fortunato Palma Esposito, Carmen Festa, Anna Poli, Pietro Tedesco, Renato Fani, Maria Chiara Monti, Donatella de Pascale, Maria Valeria D'Auria, Giovanna Cristina Varese

Legend to Supplementary figures

Fig. S1. Bayesian phylogram of Pleosporales (Dothideomycetes) taxa including the five fungal isolates (indicated as MUT) based on rDNA large subunit (LSU). Clades designation and sequences were retrieved from Gnavi et al. [14] and from GenBank. Node numbers indicate BPP over 0.60; ML bootstrap values are greater than 50%. + = strains isolated from terrestrial sources; * strains isolated from fresh water environments, mangrove swamp and salt flats; arrow indicates strains isolated from marine sources.

Fig. S2. Bayesian phylogram of Sordariomycetes taxa including the three fungal isolates (indicated as MUT) based on rDNA large subunit (LSU). Clades designation and sequences were retrieved from Gnavi et al. [14] and Tang et al.[15] and from GenBank. Node numbers indicate BPP over 0.60; ML bootstrap values are greater than 50%. + = strains isolated from terrestrial sources; * strains isolated from fresh water environments, mangrove and salt flats; arrow indicates strains isolated from marine sources.

Fig. S3. MS² spectrum of compound 1.

Fig. S4. MS² spectrum of compound 2.

Fig. S5. MS² spectrum of compound 3.

Fig. S6. MS² spectrum of compound 4.

Fig. S7. MS² spectrum of compound 6.

Fig. S8. MS² spectrum of compound 7.

Fig. S9. MS² spectrum of compound 8.

Fig. S10. MS² spectrum of compound with molecular formula C_{19}H_{39}NO_{3}

Fig. S11. MS³ data of compound with molecular formula C_{19}H_{39}NO_{3} on the daughter ions of m/z 330.30.
Table S1. Selection of the best fungi growth media antimicrobial compounds production.  
Table reports the antimicrobial activity as the percentage of inhibition of a selected target bacterium (*Burkholderia metallica* LMG 24068) in presence of the fungal extracellular extracts from the three different growth media. MeCl medium showed the best antimicrobial activity. ND: Not detected.

<table>
<thead>
<tr>
<th>MUT Code</th>
<th>Growth media</th>
<th>MeCl</th>
<th>WST30</th>
<th>PCB</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4859</td>
<td></td>
<td>55 ± 2.4</td>
<td>38 ± 1.2</td>
<td>ND</td>
</tr>
<tr>
<td>4860</td>
<td></td>
<td>50 ± 1.7</td>
<td>48 ± 2.4</td>
<td>ND</td>
</tr>
<tr>
<td>4861</td>
<td></td>
<td>65 ± 3.5</td>
<td>38 ± 4.5</td>
<td>10 ± 0.6</td>
</tr>
<tr>
<td>4865</td>
<td></td>
<td>60 ± 1.0</td>
<td>60 ± 5.7</td>
<td>ND</td>
</tr>
<tr>
<td>4883</td>
<td></td>
<td>25 ± 0.7</td>
<td>ND</td>
<td>20 ± 1.2</td>
</tr>
<tr>
<td>4885</td>
<td></td>
<td>35 ± 1.4</td>
<td>33 ± 3.2</td>
<td>25 ± 0.3</td>
</tr>
<tr>
<td>4886</td>
<td></td>
<td>30 ± 0.4</td>
<td>40 ± 4.3</td>
<td>40 ± 0.9</td>
</tr>
<tr>
<td>4966</td>
<td></td>
<td>50 ± 0.8</td>
<td>10 ± 0.2</td>
<td>ND</td>
</tr>
<tr>
<td>4979</td>
<td></td>
<td>62 ± 1.4</td>
<td>45 ± 3.5</td>
<td>38 ± 0.9</td>
</tr>
</tbody>
</table>


Table S2. Comparison of the antimicrobial activity between intracellular and extracellular extracts. Antimicrobial activity is reported as the percentage of inhibition of the selected target bacterium (*Burkholderia metallica* LMG 24068) in presence of intracellular and extracellular fungal extracts. Intracellular extracts resulted to be the most active.

<table>
<thead>
<tr>
<th>MUT Code</th>
<th>Intracellular extract</th>
<th>Extracellular extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>4859</td>
<td>70 ± 3.4</td>
<td>40 ± 3.2</td>
</tr>
<tr>
<td>4860</td>
<td>67 ± 2.1</td>
<td>33 ± 1.3</td>
</tr>
<tr>
<td>4861</td>
<td>56 ± 0.9</td>
<td>30 ± 0.5</td>
</tr>
<tr>
<td>4865</td>
<td>60 ± 2.5</td>
<td>32 ± 0.7</td>
</tr>
<tr>
<td>4883</td>
<td>54 ± 3.1</td>
<td>25 ± 0.8</td>
</tr>
<tr>
<td>4885</td>
<td>76 ± 4.3</td>
<td>33 ± 1.2</td>
</tr>
<tr>
<td>4886</td>
<td>60 ± 3.8</td>
<td>10 ± 0.6</td>
</tr>
<tr>
<td>4966</td>
<td>60 ± 2.1</td>
<td>15 ± 1.3</td>
</tr>
<tr>
<td>4979</td>
<td>60 ± 6.5</td>
<td>30 ± 2.1</td>
</tr>
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</table>
Table S3. Re-assessment of the antibiotic resistance of the four MDR bacterial strains belonging to different species. The data are reported as capacity to inhibit the microorganism growth in more than 50% (IC$_{50}$).

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>B. metallica LMG 24068</th>
<th>P. aeruginosa PA01</th>
<th>K. pneumoniae DF12SA</th>
<th>S. aureus 6538P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>&gt; 0.2</td>
<td>0.025 - 0.012</td>
<td>&lt; 0.003</td>
<td>&lt; 0.003</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>0.006 - 0.003</td>
<td>0.006 - 0.003</td>
<td>&lt; 0.003</td>
<td>&lt; 0.003</td>
</tr>
<tr>
<td>Kanamicyn</td>
<td>0.006 - 0.003</td>
<td>0.006 - 0.003</td>
<td>&lt; 0.003</td>
<td>&lt; 0.003</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.025 - 0.012</td>
<td>&lt; 0.003</td>
<td>0.006 - 0.003</td>
<td>&lt; 0.003</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>&lt; 0.003</td>
<td>0.006 - 0.003</td>
<td>&lt; 0.003</td>
<td>&lt; 0.003</td>
</tr>
</tbody>
</table>
1,3-dihydroxy-2-amino-6,17-docosadiene (1)

1,3,4-trihydroxy-2-aminooctadecane (2)

1,3-dihydroxy-2-aminooctadecane (3)

N- Acetyl-1,3,4-trihydroxy-2-aminooctadecane (4)

Aphidicolin (5)

Fusoxysporone (6)

Bis-(2-ethylhexyl)-hexanedioic acid (7)

Scopularide A (8)
Figure S3. MS² spectrum of compound 1

Figure S4. MS² spectrum of compound 2
Figure S5. MS\textsuperscript{2} spectrum of compound 3

![MS\textsuperscript{2} spectrum of compound 3]

$+ c$ ESI Full ms\textsuperscript{2} 302.30@cid35.00

Figure S6. MS\textsuperscript{2} spectrum of compound 4

![MS\textsuperscript{2} spectrum of compound 4]

$+ c$ ESI Full ms\textsuperscript{2} 360.30@cid35.00
**Figure S7.** MS² spectrum of compound 6

![MS² spectrum of compound 6](image)

**Figure S8.** MS² spectrum of compound 7

![MS² spectrum of compound 7](image)
Figure S9. MS² spectrum of compound 8

- c ESI Full ms2 672.40@cid35.00

Figure S10. MS² spectrum of compound with molecular formula C₁₉H₃₉NO₃

- c ESI Full ms2 330.30@cid35.00
**Figure S11.** MS$^3$ data on the daughter ion of m/z 330.30

![MS^3 data chart](image-url)