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

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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 nrDNAInternal 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)

100 [17] and Maximum Likelihood (ML; RAxML v.7.3.2) [18] approaches, as previously
101 described [14]. Bayesian Posterior Probability (BPP) values over 0.6 (with MLB over 50%)
102 are reported in the resulting trees.
103
104 **Fungal growth conditions**
105 Preliminary growth condition tests were performed in order to define the most
106 effective and appropriate medium to induce the production of bioactive secondary metabolites
107 in the selected fungal strains. Each fungal strain was inoculated in duplicate by 10 agar plugs
108 of 5 mm diameter cut from the edge of actively growing culture onto malt extract agar in 150
109 ml flasks containing 100 ml of three different media: PCB (10 g of crushed potatoes and 10 g
110 of crushed carrots in 1 L of ddH_2O), MeCl (20 g malt extract, 17 g NaCl in 1 L of ddH_2O) and
111 WST30 (10 g glucose monohydrate, 5 g soya peptone, 3 g malt extract, 3 g yeast extract, 30 g
112 NaCl). Flasks were incubated in the dark at 24°C and rotated at 150 rpm. The broth and
113 mycelium of each strain were collected after 2 and 4 weeks and submitted to an extraction
114 procedure for the preliminary bio-chemical analysis (see below). The MeCl medium and 4
115 week-incubation were selected as the best conditions (24°C in the dark). Hence, each fungus
116 was inoculated (100 agar plugs of 5 mm diameter) in 2 L flasks containing 1.5 L of MeCl,
117 which was incubated in the dark at 24°C, at 180 rpm for 4 weeks.
118 **Extract preparation**
119 Samples were centrifuged at 11,200 x rcf for 30 min at 4 °C and filtrated in order to
120 separate the mycelium from the culture broth. Supernatants were extracted with ethyl acetate
121 (EtOAc) and the resulting extracts were dried-out by using a Rotavapor, weighed, solubilized
122 in dimethyl sulfoxide (DMSO, 100%) at a final concentration of 100 mg/mL and stored at -
123 20°C. The presence of antimicrobial compounds in the mycelia was also evaluated. In order
124 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 N_2/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 IC_{50} 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 ddH_2O), 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 tetramethylsilane (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_2O 10% acetonitrile (ACN) 0.1% formic acid (FA) and mobile phase B was 10% H_2O 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^nanalysis 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 Biatriosporaceae 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_{50} values. The resistance of each strain to Ampicillin, Chloramphenicol, Kanamycin, Tetracycline and Trimethoprim was confirmed and IC_{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 *B. metallica* (IC_{50} 0.5-0.25 mg/mL) and *S. aureus,* and was the only one to show, by both EtOAc and acetone extracts, an inhibitory effect against *P. aeruginosa.* Both extracts from MUT 4865 were able to inhibit *B. metallica* and *S. aureus* (IC_{50} 0.5-0.25) and the EtOAc extracts also showed inhibition against *K. pneumoniae*. No effects were observed against *P. aeruginosa.* The extract from MUT 4979 showed antimicrobial activity against three out of the four pathogens (IC_{50} 1.0-0.25), with the exception of *K. pneumoniae.* Extracts of MUT 4859, 4860, and 4966 only showed a significant activity against *B. metallica* and *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.

225 Overall, the most promising strains were MUT 4865, 4979 and 4861,which exhibited
226 the highest degree of antibacterial activity.

227 **Secondary Metabolite Analyses**

228 Based on the results of the preliminary pharmacologic, spectroscopic and
229 chromatographic screening, the extracts of MUT 4865 and MUT4861 were selected for the
230 chemical profiling and were analyzed by LC-HRMS. Other strains did not produce detectable
231 amounts of secondary metabolites under cultivation conditions and, therefore, revealing their
232 potential of secondary metabolite production will require further investigation.

233 *Beauveria bassiana* MUT 4865**:** both acetone and EtOAc extracts were subjected to
234 HRESIMS analysis (**Fig. 1A**). Compound **1** analyzed for $C_{22}H_{43}O_2N$ by HRMS analysis
235 (calculated for $C_{22}H_{43}NO_2Na$: 376.3192, found $[M + Na]^+$: 376.3195). In the MS^2 spectrum
236 (Supplementary materials **Fig. S3**), the sequential loss of one ammonia and two neutral water
237 molecules indicated the presence of one amino and two hydroxyl groups. The planar structure
238 of this compound was deduced from the analysis of the MS^3 spectrum, which showed a
239 fragmentation pattern compatible with the localization of the two double bonds at the unusual
240 positions of 6 and 17, revealing that it corresponded to the long chain sphingadienine (**Fig. 2**).
241 Therefore a 1,3-dihydroxy-2-amino-6,17-docosadiene structure was tentatively proposed.
242 Assignment of the relative configuration of the two contiguous stereogenic centers, as well as
243 of the two double bonds would require isolation of the compound from a large-scale
244 cultivation batch of the fungal strain.

245 As shown in **Fig. 1B**, the acetone extract did not contain a detectable amount of
246 compound **1,** whereas some sphingosine compounds were detected, such as phytosphingosine
247 (**2**), dihydrosphingosine (**3**) and phytoceramide C2 (**4**). The MS^2 pattern analysis (**Table 3** and
248 Supplementary materials **Fig. S4-S6**) leads to a straightforward assignment of a planar
249 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 enthomopathogenic 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

300 producing antimicrobial compounds from new taxonomic entities that have never been
301 previously explored.

302 The most promising fungal strains were MUT 4865, 4979 and 4861, which exhibited
303 the highest degree of antibacterial activity. MUT 4865, identified as *B. bassiana,*
304 representatives of which are well-known producers of insecticidals and antimicrobials [36],
305 showed a strong activity against all the pathogens tested. For *K. petricola* (MUT 4979), this is
306 the first report of an antimicrobial activity exhibited by the fungal extracts from this species.
307 Further studies are necessary, considering that the class this organism belongs to
308 (Eurotiomycetes) includes several species (e.g. *Aspergillus* spp., *Paecilomyces* spp.,
309 *Penicillum* spp.) that have been reported to be a source of many antimicrobial metabolites [37,
310 38].

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

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

319 In particular, the long chain sphingadienine 2-aminodocosa-6,17-dien-1,3-diol has
320 never been described as a free base or as a component of polar lipids from natural sources.
321 The related docosa-4,15-sphingadienine and 4-hydroxy-docosa-15-sphingenine have been
322 reported as components in sphingophosphonolipids from the marine gastropod *Turbo*
323 *cornutus* [41]. Noteworthy, recent years have witnessed an ever-increasing interest towards
324 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, aphidilcolin 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 Microascacea 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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521 **Legends to figures**
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523 **Fig. 1.** ESI positive mode base peak chromatograms of the active samples MUT 4865 EtOAc
524 extract (panel **A**), Acetone extract (panel **B**) and MUT 4861 Acetone extract (panel **C**).
525 Numbers above the peaks identify the metabolites listed in **Tables 2** and **3**.

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

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530 **Fig. 3.** Chemical structures of secondary metabolites (**1**-**8**) identified by LC-HRMS in the
531 bioactive extracts of *Beauveria bassiana* MUT 4865 and MUT 4861.

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550 **Table 1.** MUT code, taxonomic assessment of sterile mycelia isolated from *F. petiolata* and
551 GenBank accesion numbers.

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MUT Code	Fungal taxa	GenBank accession number ITS and LSU
4883	***Biatriospora* sp.**	KR014352 KP671728
4865	***Beauveria bassiana*** (Bals.-Criv.) Vuill.	KR014380 KP671729
4860	***Massarina* sp.**	KR014362 KP671730
4885	**Microascacea sp. 1**	KR014356 KP671717
4861	**Microascacea sp. 2**	KR014360 KP671746
4859	**Roussoellacea sp. 1**	KR014355 KP671716
4886	**Roussoellacea sp. 2**	KR014358 KP671720
4966	**Roussoellacea sp. 3**	KR014366 KP671740
4979	***Knufia petricola*** (U. Wollenzien & de Hoog) Gorbushina & Gueidan	KR014376 KP671749

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569 **Table 2.** Antimicrobial activity of the fungal intracellular extracts *vs* four bacterial strains
570 belonging to different species. The data are reported as capacity to inhibit the microorganisms
571 growth in more than 50% (IC_{50}). Growth in the presence of 2% DMSO was considered as
572 100% growth. ND: Not detected.

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Fungi MUT CODE	IC_{50} (mg/mL)							
	***B. metallica* LMG 24068**		***P. aeruginosa* PA01**		***K. Pneumoniae* DF12SA**		***S. aureus* 6538P**	
	Ethyl acetate	**Acetone**	**Ethyl acetate**	**Acetone**	**Ethyl acetate**	**Acetone**	**Ethyl acetate**	**Acetone**
4859	0.5 - 0.25	> 2.0	> 2.0	> 2.0	> 2.0	> 2.0	1.0 - 0.5	> 2.0
4860	0.5 - 0.25	0.5 - 0.25	> 2.0	> 2.0	> 2.0	> 2.0	2.0 - 1.0	> 2.0
4861	0.5 - 0.25	0.5 - 0.25	2.0 - 1.0	1.0 - 0.5	> 2.0	2.0 - 1.0	1.0 - 0.5	ND
4865	0.5 - 0.25	0.5 - 0.25	> 2.0	> 2.0	1.0 - 0.5	> 2.0	0.5 - 0.25	0.5 - 0.25
4979	1.0 - 0.5	ND	1.0 - 0.5	ND	> 2.0	ND	0.5 - 0.25	ND
4966	1.0 - 0.5	ND	> 2.0	ND	> 2.0	ND	1.0 - 0.5	ND
4885	2.0 - 1.0	ND	> 2.0	ND	> 2.0	ND	2.0 - 1.0	ND
4886	2.0 - 1.0	ND	> 2.0	ND	> 2.0	ND	2.0 - 1.0	ND
4883	2.0 - 1.0	ND	2.0 - 1.0	ND	> 2.0	ND	2.0 - 1.0	ND

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592 **Table 3.** Annotated peaks observed in the chromatograms of the EtOAc and Acetone extracts
593 of *Beauveria bassiana* MUT 4865
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RT (min)	MS and MS/MS	Suggested MF	Proposed structure
23.20	376.3195 $[M+Na]^+$ (Δppm: 1.049) MS^2 (**Fig. S3**): 359.29, 341.28; MS^3 see **Fig. 2**	$C_{22}H_{43}NO_2$	2-aminodocosa-6,17-dien-1,3-diol (**1**)
28.32	318.30015 (Δppm: -0.379) MS^2 (**Fig. S4**) :300.29, 282.29, 265.33	$C_{18}H_{39}NO_3$	2-aminooctadecan-1,3,4-triol (4-hydroxysphiganine or phytosphingosine) (**2**)
29.11	302.30543 (Δppm: 0.245) MS^2 (**Fig. S5**): 284.29, 266.31, 249.26	$C_{18}H_{39}NO_2$	2-aminooctadecan-1,3-diol (dihydrosphingosine) (**3**)
30.03	360.31079 (Δppm: -0,126) MS^2 (**Fig. S6**) :342.31,324.32, 300.31, 264.30, 212.19	$C_{20}H_{41}NO_4$	N-[1,3,4-trihydroxyoctadecan-2-yl]acetamide (phytoceramide C2) (**4**)
45.65	339.25320 (Δppm: - 0.876)	$C_{20}H_{34}O_4$	Aphidicolin (**5**)
54.04	287.23634 (Δppm: 0.584) MS^2 (**Fig. S7**): 269.23, 203.14, 175.11	$C_{20}H_{30}O$	Fusoxysporone (**6**)
60.38	395.3309 (Δppm: 0.145)	$C_{28}H_{42}O$	Ergosta-5,7,22-trien-3-β-ol (ergosterol)
62.89	393.3153 (Δppm: 0.401)	$C_{28}H_{40}O$	Ergostane derivative
66.49	371.31453 (Δppm: -1.056) MS^2 (**Fig. S8**): 259.01, 240.70, 146.9, 128.9, 110.99	$C_{22}H_{42}O_4$	Bis(2-ethylhexyl) hexanedioic acid (**7**)

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606 **Table 4.** Annotated peaks observed in the chromatograms of the Acetone extract of
607 Microascacea sp.2 MUT 4861.
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RT (min)	MS and MS^n	Suggested MF	Proposed structure
31.52	318.30002 (Δppm -0.756) MS^2 (**Fig. S4**): 300.29, 282.29, 265.33	$C_{18}H_{39}NO_3$	2-amino-octadecane- 1,3,4- triol (4-hydroxysphiganine or phytosphingosine) (**2**)
34.29	330.30024 (Δppm -0.031) MS^2 (**Fig. S10**): 312.26, 294.33, 282.32, 256.32 [MS^3(@ 294.33)] (Fig. **S11**): 266.33, 168.18, 154.07, 140.11, 133.01, 126.0, 111.96, 97.94)	$C_{19}H_{39}NO_3$	
49.05	672.43291 (Δppm-0.166) MS^2 (**Fig. S9**) 654.5, 525.3, 507.2, 454.2, 436.2, 323.1	$C_{36}H_{57}N_5O_7$	Scopularide A (**8**)
58.14	409.3101 (Δppm 0)	$C_{28}H_{40}O_2$	Ergostane derivative
59.94	393.3154 (Δppm 0)	$C_{28}H_{40}O$	Ergostane derivative
65.6	395.3307 (Δppm 0)	$C_{28}H_{42}O$	Ergosterol
73.06	371.31576 (Δppm 0) MS^2 (**Fig. S8**): 259.01, 240.70, 146.9, 128.9, 110.99	$C_{22}H_{42}O_4$	Bis(2-ethylhexyl) hexanedioic acid (**7**)
77.20	377.32019 (Δppm 0)	$C_{28}H_{40}$	Ergosta-3,5,7,9(11),22-pentaene

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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 Sordariomycestes 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. MS^2 spectrum of compound **1**.

Fig. S4. MS^2 spectrum of compound **2**.

Fig. S5. MS^2 spectrum of compound **3**.

Fig. S6. MS^2 spectrum of compound **4**.

Fig. S7. MS^2 spectrum of compound **6**.

Fig. S8. MS^2 spectrum of compound **7**.

Fig. S9. MS^2 spectrum of compound **8**.

Fig. S10. MS^2 spectrum of compound with molecular formula$C_{19}H_{39}NO_3$

Fig. S11. MS^3 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.

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

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.

MUT Code	Intracellular extract	Extracellular extract
4859	70 ± 3.4	40 ± 3.2
4860	67 ± 2.1	33 ±1.3
4861	56 ± 0.9	30 ± 0.5
4865	60 ± 2.5	32 ± 0.7
4883	54 ± 3.1	25 ± 0.8
4885	76 ± 4.3	33 ± 1.2
4886	60 ± 3.8	10 ± 0.6
4966	60 ± 2.1	15 ± 1.3
4979	60 ± 6.5	30 ± 2.1

725 **Table S3. Re-assessment of the antibiotic resistance of the four MDR bacterial strains**
726 **belonging to different species.** The data are reported as capacity to inhibit the
727 microorganism growth in more than 50% (IC_{50}).

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Antibiotic	IC_{50} (mg/mL)			
	B. metallica **LMG 24068**	***P. aeruginosa*** **PA01**	***K. pneumoniae*** **DF12SA**	***S. aureus*** **6538P**
Ampicillin	> 0.2	0.025 -0.012	< 0.003	< 0.003
Chloramphenicol	0.006 - 0.003	0.006 - 0.003	< 0.003	< 0.003
Kanamicyn	0.006 - 0.003	0.006 - 0.003	< 0.003	< 0.003
Tetracycline	0.025 -0.012	< 0.003	0.006 - 0.003	< 0.003
Trimethoprim	< 0.003	0.006 - 0.003	< 0.003	< 0.003

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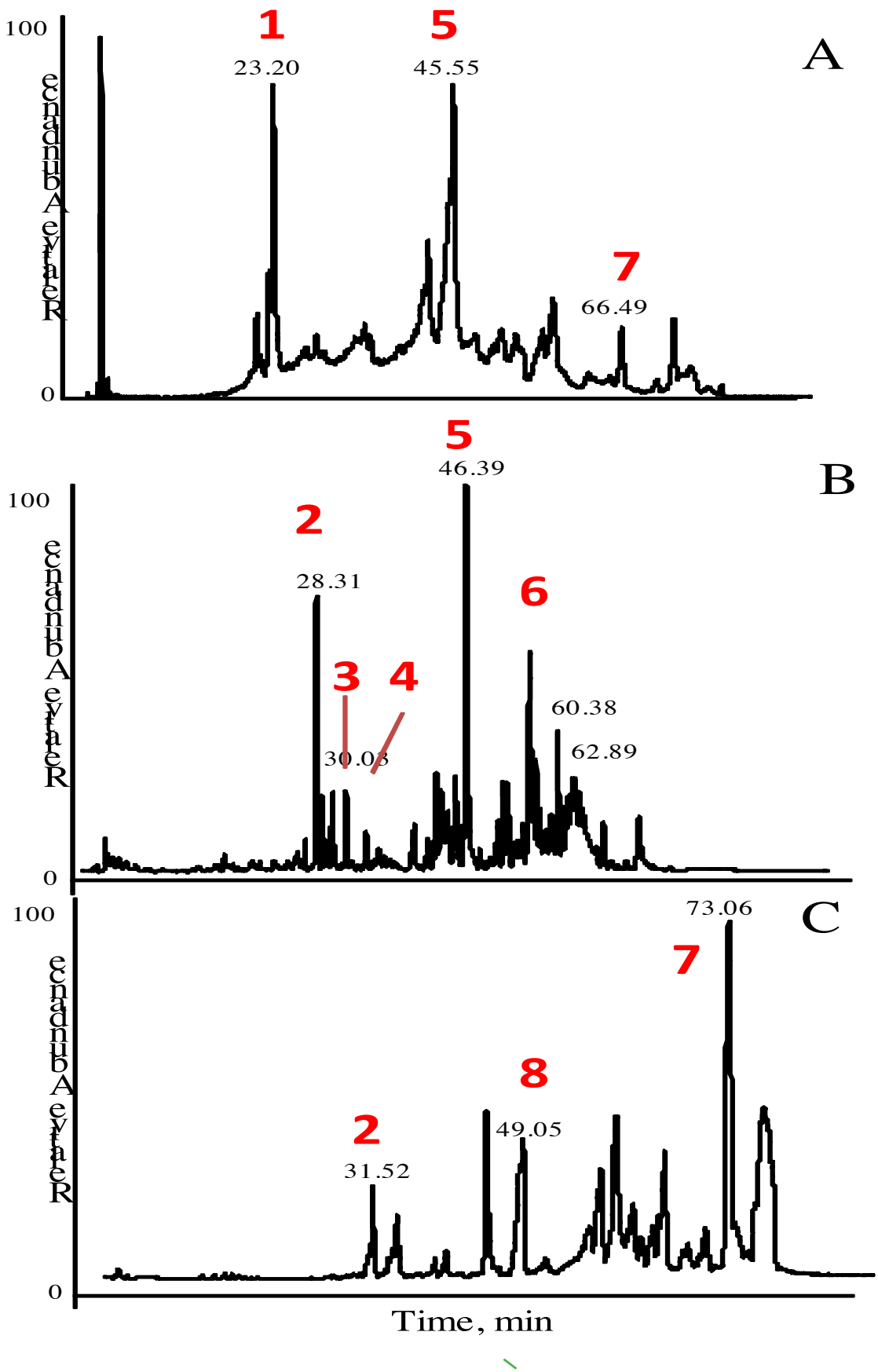
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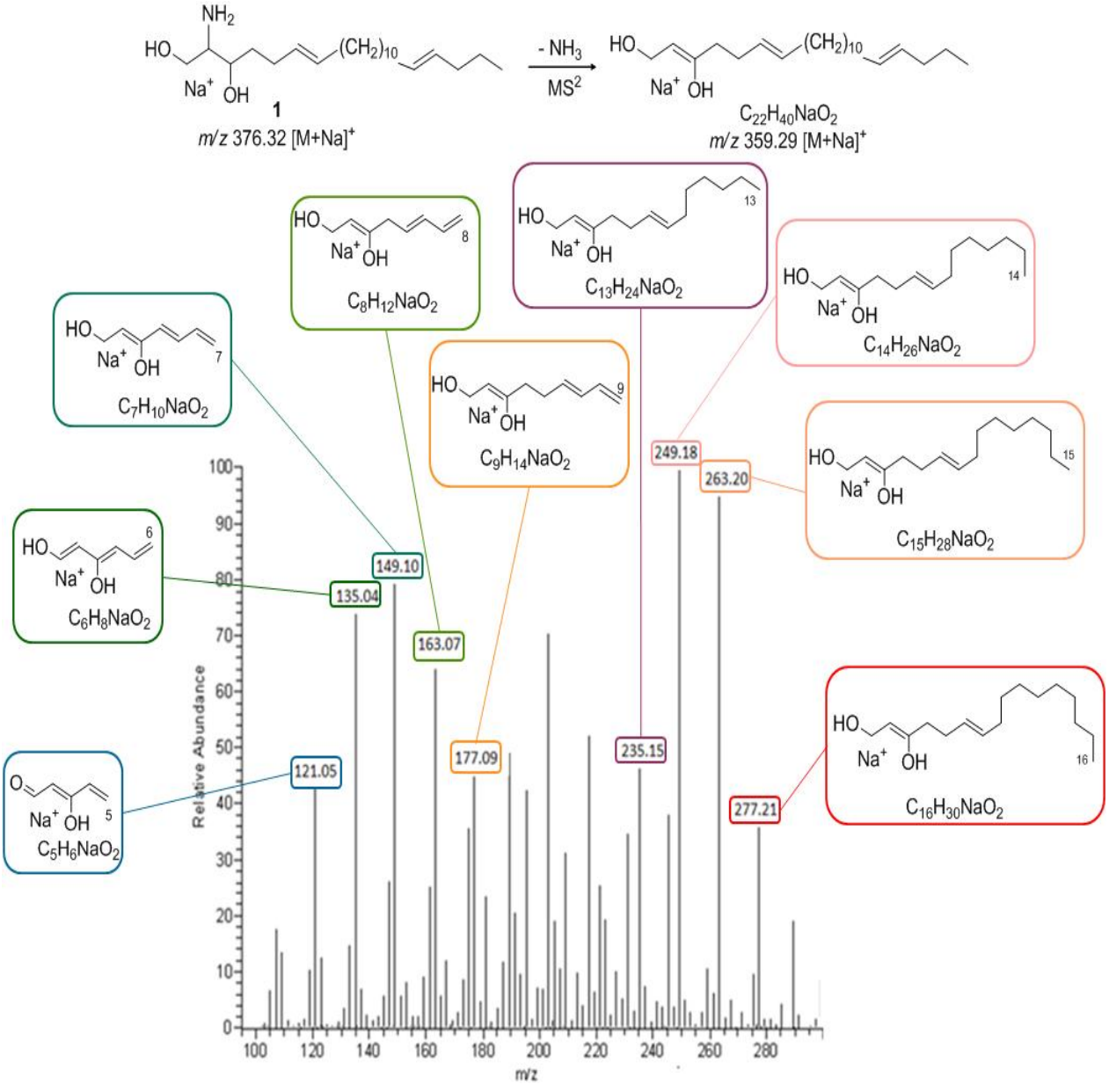
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C6H8NaO2
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C5H6NaO2
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163.07
177.09
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249.18
263.20
235.15
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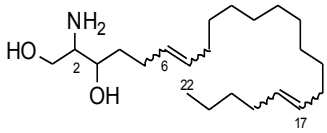
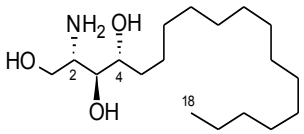
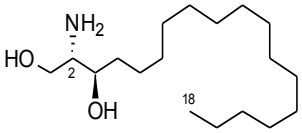


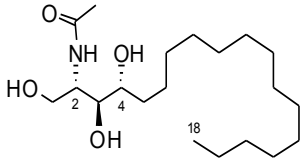
1,3-dihydroxy-2-amino-6,17-docosadiene (**1**)



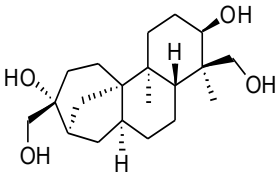
1,3,4-trihydroxy-2-aminooctadecane (**2**)



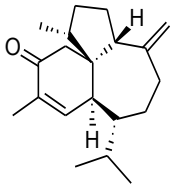
1,3-dihydroxy-2-aminooctadecane (**3**)



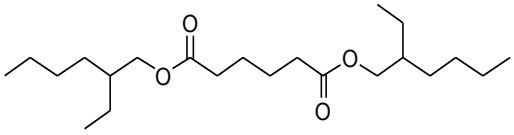
N- Acetyl-1,3,4-trihydroxy-2-aminooctadecane (**4**)



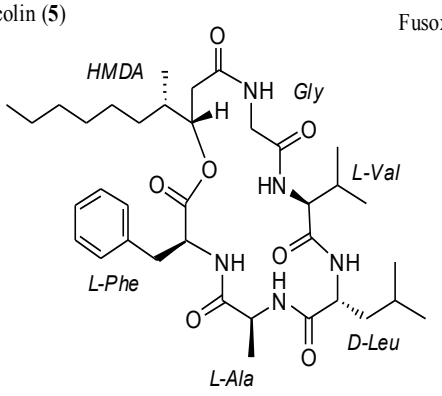
Aphidicolin (**5**)



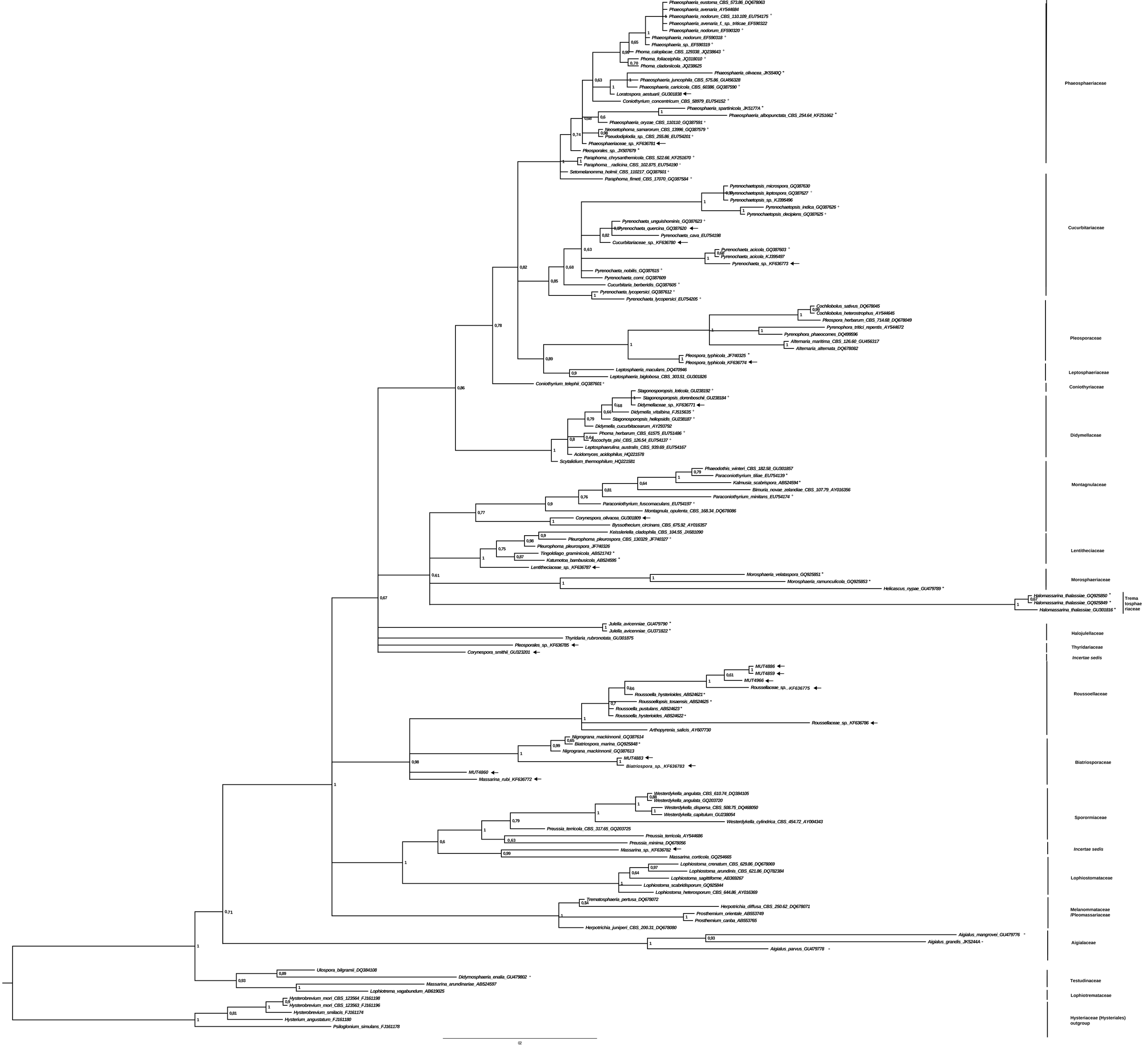
Fusoxysporone (**6**)



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



Scopularide A (**8**)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Phaeosphaeria_nodorum_EF590320 +
Phaeosphaeria_nodorum_EF590318 +
Phaeosphaeria_sp._EF590319 +
Phoma_caloplacae_CBS_129338_JQ238643 +
Phoma_foliaceiphila_JQ318010 +
Phoma_cladoniicola_JQ238625
Phaeosphaeria_olivacea_JK5540Q *
Phaeosphaeria_juncophila_CBS_575.86_GU456328
Phaeosphaeria_caricicola_CBS_60386_GQ387590 +
Loratospora_aestuarii_GU301838
Coniothyrium_concentricum_CBS_58979_EU754152 +
Phaeosphaeria_spartinicola_JK5177A *
Phaeosphaeria_albopunctata_CBS_254.64_KF251662 *
Phaeosphaeria_oryzae_CBS_110110_GQ387591 +
Neosetophoma_samarorum_CBS_13996_GQ387579 +
Pseudodidymella_sp._CBS_255.86_EU754201 +
Phaeosphaeriaceae_sp._KF636781
Pleosporales_sp._JX507679 *
Paraphoma_chrysanthemicola_CBS_522.66_KF251670 +
Paraphoma__radicina_CBS_102.875_EU754190 +
Setomelanomma_holmii_CBS_110217_GQ387601 +
Paraphoma_fimeti_CBS_17070_GQ387584 +
Pyrenochaetopsis_microspora_GQ387630
Pyrenochaetopsis_leptospora_GQ387627 +
Pyrenochaetopsis_sp._KJ395496
Pyrenochaetopsis_indica_GQ387626 +
Pyrenochaetopsis_decipiens_GQ387625 +
Pyrenochaeta_unguishominis_GQ387623 +
Pyrenochaeta_quercina_GQ387620
Pyrenochaeta_cava_EU754198
Cucurbitariaceae_sp._KF636780
Pyrenochaeta_acicola_GQ387603 +
Pyrenochaeta_acicola_KJ395497
Pyrenochaeta_sp._KF636773
Pyrenochaeta_nobilis_GQ387615 +
Pyrenochaeta_corni_GQ387609
Cucurbitaria_berberidis_GQ387605 +
Pyrenochaeta_lycopersici_GQ387612 +
Pyrenochaeta_lycopersici_EU754205 +
Cochliobolus_sativus_DQ678045
Cochliobolus_heterostrophus_AY544645
Pleospora_herbarum_CBS_714.68_DQ678049
Pyrenophora_tritici_repentis_AY544672
Pyrenophora_phaeocomes_DQ499596
Alternaria_maritima_CBS_126.60_GU456317
Alternaria_alternata_DQ678082
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Pleospora_typhicola_KF636774
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Coniothyrium_telephii_GQ387601 +
Stagonosporopsis_loticola_GU238192 +
Stagonosporopsis_dorenboschii_GU238184 +
Didymellaceae_sp._KF636771
Didymella_vitalbina_FJ515635 +
Stagonosporopsis_heliopsidis_GU238187 +
Didymella_cucurbitacearum_AY293792
Phoma_herbarum_CBS_61575_EU751486 +
Ascochyta_pisi_CBS_126.54_EU754137 +
Leptosphaerulina_australis_CBS_939.69_EU754167
Acidomyces_acidophilus_HQ221578
Scytalidium_thermophilum_HQ221581
Phaeodothis_winteri_CBS_182.58_GU301857
Paraconiothyrium_tiliae_EU754139 *
Kalmusia_scabrispora_AB524594 *
Bimuria_novae_zelandiae_CBS_107.79_AY016356
Paraconiothyrium_minitans_EU754174 +
Paraconiothyrium_fuscomaculans_EU754197 +
Montagnula_opulenta_CBS_168.34_DQ678086
Corynespora_olivacea_GU301809
Byssothecium_circinans_CBS_675.92_AY016357
Keissleriella_cladophila_CBS_104.55_JX681090
Pleurophoma_pleurospora_CBS_130329_JF740327 +
Pleurophoma_pleurospora_JF740326
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Katumotoa_bambusicola_AB524595 *
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Morosphaeria_ramunculicola_GQ925853 *
Helicascus_nypae_GU479789 *
Halomassarina_thalassiae_GQ925850 *
Halomassarina_thalassiae_GQ925849 *
Halomassarina_thalassiae_GU301816 *
Julella_avicenniae_GU479790 *
Julella_avicenniae_GU371822 *
Thyridaria_rubronotata_GU301875
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Corynespora_smithii_GU323201
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MUT4859
MUT4966
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Roussoellopsis_tosaensis_AB524625 *
Roussoella_pustulans_AB524623 *
Roussoella_hysterioides_AB524622 *
Roussellaceae_sp._KF636786
Arthopyrenia_salicis_AY607730
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Biatriospora_marina_GQ925848 *
Nigrograna_mackinnonii_GQ387613
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Biatriospora_sp._KF636783
MUT4860
Massarina_rubi_KF636772
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Westerdykella_angulata_GQ203720
Westerdykella_dispersa_CBS_508.75_DQ468050
Westerdykella_capitulum_GU238054
Westerdykella_cylindrica_CBS_454.72_AY004343
Preussia_terricola_CBS_317.65_GQ203725
Preussia_terricola_AY544686
Preussia_minima_DQ678056
Massarina_sp._KF636782
Massarina_corticola_GQ254665
Lophiostoma_crenatum_CBS_629.86_DQ678069
Lophiostoma_arundinis_CBS_621.86_DQ782384
Lophiostoma_sagittiforme_AB369267
Lophiostoma_scabridisporum_GQ925844
Lophiostoma_heterosporum_CBS_644.86_AY016369
Trematosphaeria_pertusa_DQ678072
Herpotrichia_diffusa_CBS_250.62_DQ678071
Prosthemium_orientale_AB553749
Prosthemium_canba_AB553765
Herpotrichia_juniperi_CBS_200.31_DQ678080
Aigialus_mangrovei_GU479776 *
Aigialus_grandis_JK5244A *
Aigialus_parvus_GU479778 *
Ulospora_bilgramii_DQ384108
Didymosphaeria_enalia_GU479802 *
Massarina_arundinariae_AB524597
Lophiotrema_vagabundum_AB619025
Hysterobrevium_mori_CBS_123564_FJ161198
Hysterobrevium_mori_CBS_123563_FJ161196
Hysterobrevium_smilacis_FJ161174
Hysterium_angustatum_FJ161180
Psiloglonium_simulans_FJ161178
Phaeosphaeriaceae
Cucurbitariaceae
Pleosporaceae
Leptosphaeriaceae
Coniothyriaceae
Didymellaceae
Montagnulaceae
Lentitheciaceae
Morosphaeriaceae
Trematosphaeriaceae
Halojulellaceae
Thyridariaceae
Incertae sedis
Roussoellaceae
Biatriosporaceae
Sporormiaceae
Incertae sedis
Lophiostomataceae
Melanommataceae /Pleomassariaceae
Aigialaceae
Testudinaceae
Lophiotremataceae
Hysteriaceae (Hysteriales) outgroup
02

Hypocrea_schweinitzii_CBS_243.63_U47833
Trichoderma_reesei_AF510497
Aphysiostroma_stercorarium_AF543792
Trichoderma_atroviride_EF591763
Hypomyces_chlorinigenus_AF213027
Hypomyces_australis_AM779860 +
Hypomyces_polyporinus_CBS_168.89_AF543793
Hypomyces_orthosporus_AF160241
Hypomyces_aurantius_AF160230
Hypocrea_pallida_U00740
Hypomyces_siamensis_AJ583484 +
Hypomyces_subiculosus_AJ459309
Balansia_henningsiana_AY489715 +
Claviceps_paspali_CBS_110.22_U47826
Chamaeleomyces_granulomatis_HM635077
Chamaeleomyces_granulomatis_JN990695 +
Cordyceps_gunnii_HM119590
Pochonia_bulbillosa_JQ780662 +
Beauveria_bassiana_EU334676 +
Beauveria_bassiana_EU334679 +
Beauveria_bassiana_JF429894
MUT_4865
Beauveria_sp.HM135177
Beauveria_caledonica_AF339520
Cordyceps_spegazzinii_DQ196435
Cordyceps_taishanensis_JN411084
Cordyceps_militaris_AF327374
Cordyceps_militaris_HM135162
Ascopolyporus_philodendrus_AY886545 +
Hypocreales
Nitschkia_grevillei_AY346294
Acanthonitschkea_argentinensis_AY695261
Fracchiaea_broomeana_AY695268
Bertia_moriformis_AY695261
Chaetosphaerella_phaeostroma_AY346346274
Coronophorales
Chaunopycnis_alba_HM595579
Chaunopycnis_sp._HM595578
Ophiocordyceps_sinensis_AB067710
Valetoniellopsis_laxa_AY015635
Ophiocordyceps_sinensis_AB067709
Ophiocordyceps_crassispora_AB067706
Pseudonectria_rousseliana_U17416
Nectria_cinnabarina_AF193237
Clonostachys_rosea_DQ862027 +
Bionectria_pityrodes_AY489728 +
Hydropisphaera_erubescens_AY545726
Stilbocrea_macrostoma_AY489725 +
Melanopsamma_pomiformis_AY489709 +
Myrothecium_inundatum_AY489731 +
Hypocreales
Microascus_trigonosporus_U47835
Microascus_cirrosus_CBS_217.31_AF275539
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Petriella_setifera_DQ470969
Pseudallescheria_apiosperma_FJ345358
Doratomyces_stemonitis_DQ836907
MUT_4861
Microascales
Neptunella_longirostris_AF539473
Halosphaeria_appendiculata_CBS_197.60_U46885
Lignincola_laevis_U46890
Nais_inornata_AF539476
Aniptodera_chesapeakensis_U46882
Halosarpheia_fibrosa_U46886
Corollospora_maritima_CBS_264.59_AF491260
Halosphaeriales
Farrowia_seminuda_AF286410
Chaetomium_longicolleum_FJ666365 +
Thielavia_terrestris_JX280761
Chaetomidium_trichorobustum_FJ666352 +
Zopfiella_ebriosa_CBS_11.75_AY346305
Chaetomidium_arxii_FJ666359 +
Chaetomidium_galaicum_FJ666360 +
Chaetomidium_leptoderma_FJ666353 +
Chaetomidium_galaicum_FJ666361 +
Chaetomium_globosum_AY545729
Chaetomium_globosum_U47825
Chaetomidium_subfimeti_KJ666357 +
Chaetomidium_fimeti_FJ666351 +
Chaetomidium_fimeti_FJ666358 +
Chaetomidium_pilosum_FJ666356 +
Chaetomium_elatum_DQ368628
Sordariales
Sordaria_macrospora_AY346301
Sordaria_fimicola_CBS_723.96_AF132330
Neurospora_crassa_CBS_709.71_AF286411
Gelasinospora_tetrasperma_DQ470980
Neurospora_crassa_FJ360521
Schizothecium_curvisporum_AY346300
Podospora_fimbriata_CBS_144.54_AY780075
Cercophora_caudata_CBS_606.72_AY999113
Bombardia_bombarda_AY780053
Lasiosphaeria_ovina_CBS_958.72_AF064643
Cercophora_septentrionalis_U47823
Thielavia_australiensis_JQ067899
Melanocarpus_albomyces_JQ067902
Thielavia_subthermophila_HM448442 +
Myceliophthora_sepedonium_FJ666364 +
Remersonia_thermophila_JQ067901
Myceliophthora_hinnulea_JQ067900
Myceliophthora_fergusii_JQ067896
Sordariomycetidae
Incertae sedis
Camarops_ustulinoides_AY346267
Camarops_tubulina_AY346266
Camarops_petersii_AY346265
Camarops_amorpha_AY780054
Camarops_microspora_CBS_649.92_AY083821
Boliniales
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Melanochaeta_hemipsila_AY346292
Chaetosphaeriales
Coniochaeta_sp._HM595581
Poroconiochaeta_discoidea_AY346297
Coniochaetidium_savoryi_AY346276
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Apioplagiostoma_aceriferum_CBS781.79_AF277129
Discula_destructiva_AF362568 +
Melanconis_marginalis_AF408373 +
Cryptodiaporthe_corni_CBS_245.90_AF408343 +
Cryphonectria_parasitica_AF277132
Diaporthe_phaseolorum_CBS_435.87_U47830
Gnomonia_ribicola_CBS_115443_DQ368626
Valsa_ceratosperma_AF408387 +
Diaporthales
Pseudohalonectria_lignicola_AY346299
Ophioceras_tenuisporum_AY346295
Gaeumannomyces_graminis_AF362557 +
Incertae sedis
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Ophiostoma_piliferum_CBS_129.32_AY281094
Ophiostomatales
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Lindra_thalassiae_AF195633
Lulworthia_grandispora_DQ5222856
Lulworthia_fucicola_AF491270
Lulworthiales
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Apiospora_setosa_DQ368631
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Amphisphaeria_umbrina_AF452029
Xylariales
Xylariomycetidae
Pleospora_herbarum_CBS191.86_AF382386
Pleosporales (Dothideomycetidae)
outgroup
Hypocreomycetidae
02

Figure S3. MS^2 spectrum of compound **1**

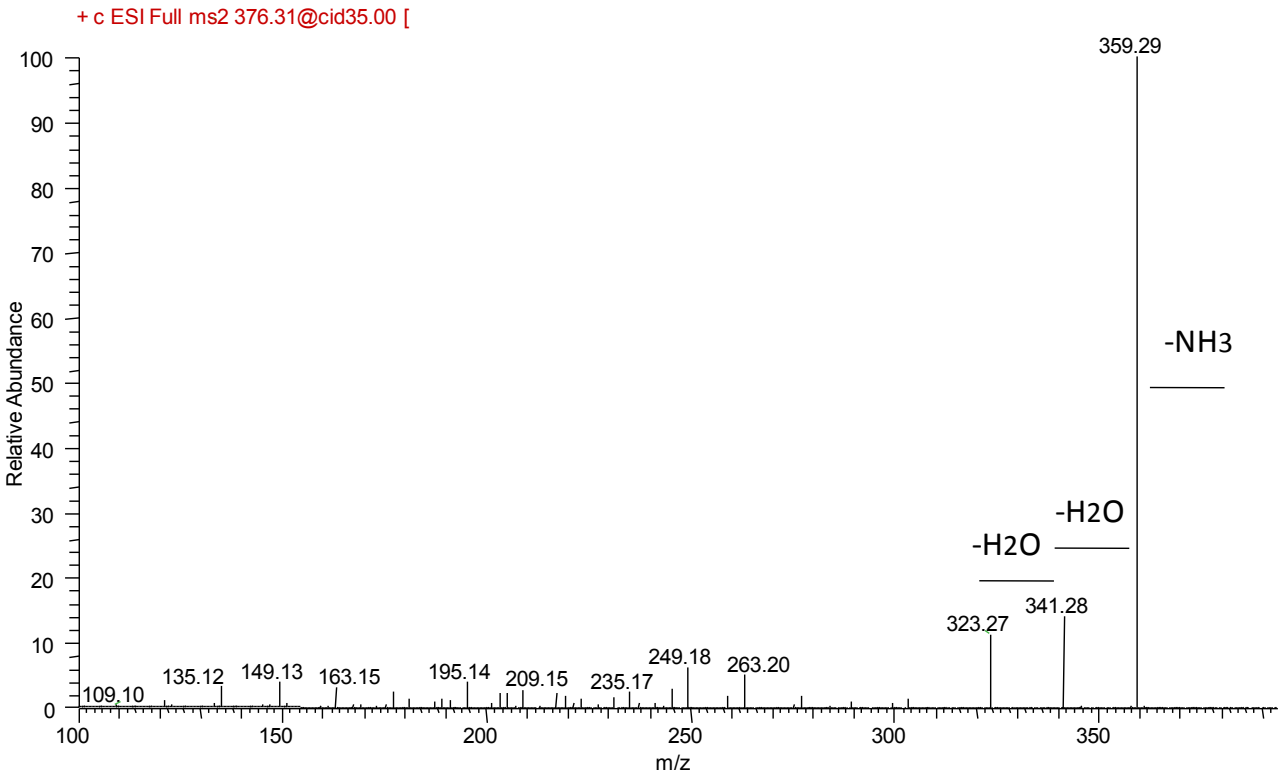

Figure S4. MS^2 spectrum of compound **2**

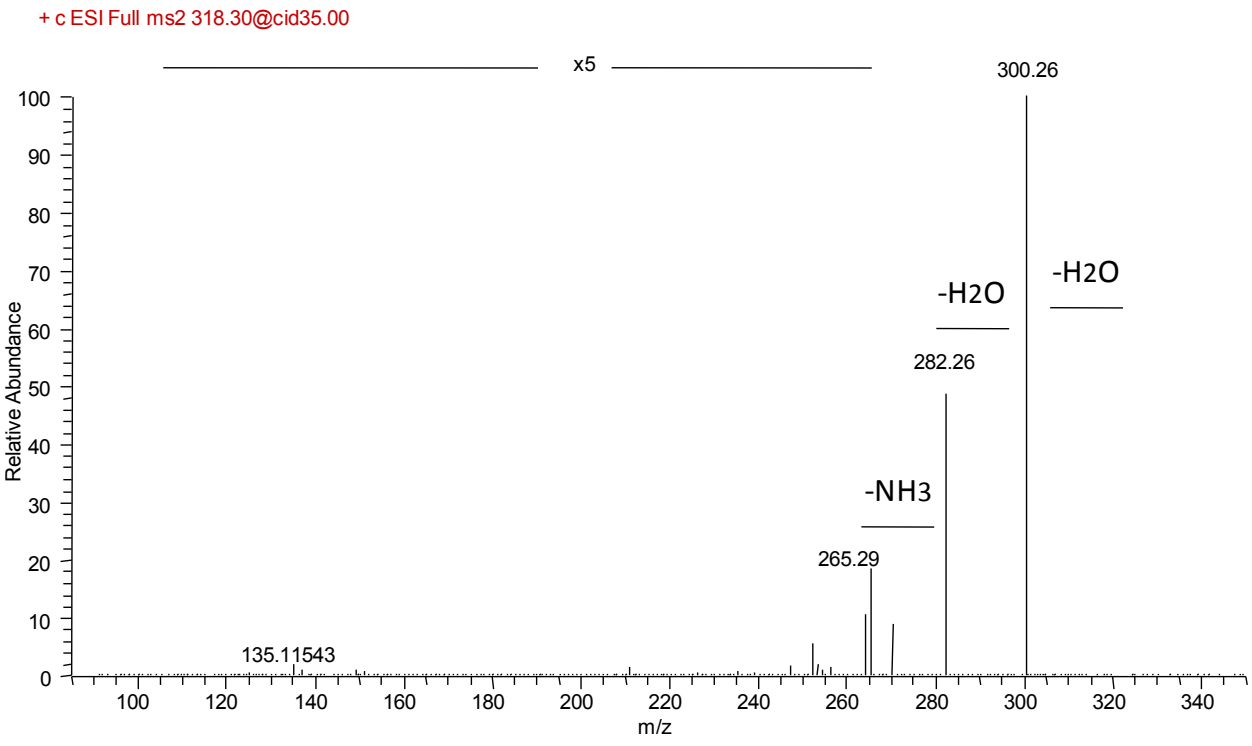

Figure S5. MS^2 spectrum of compound **3**

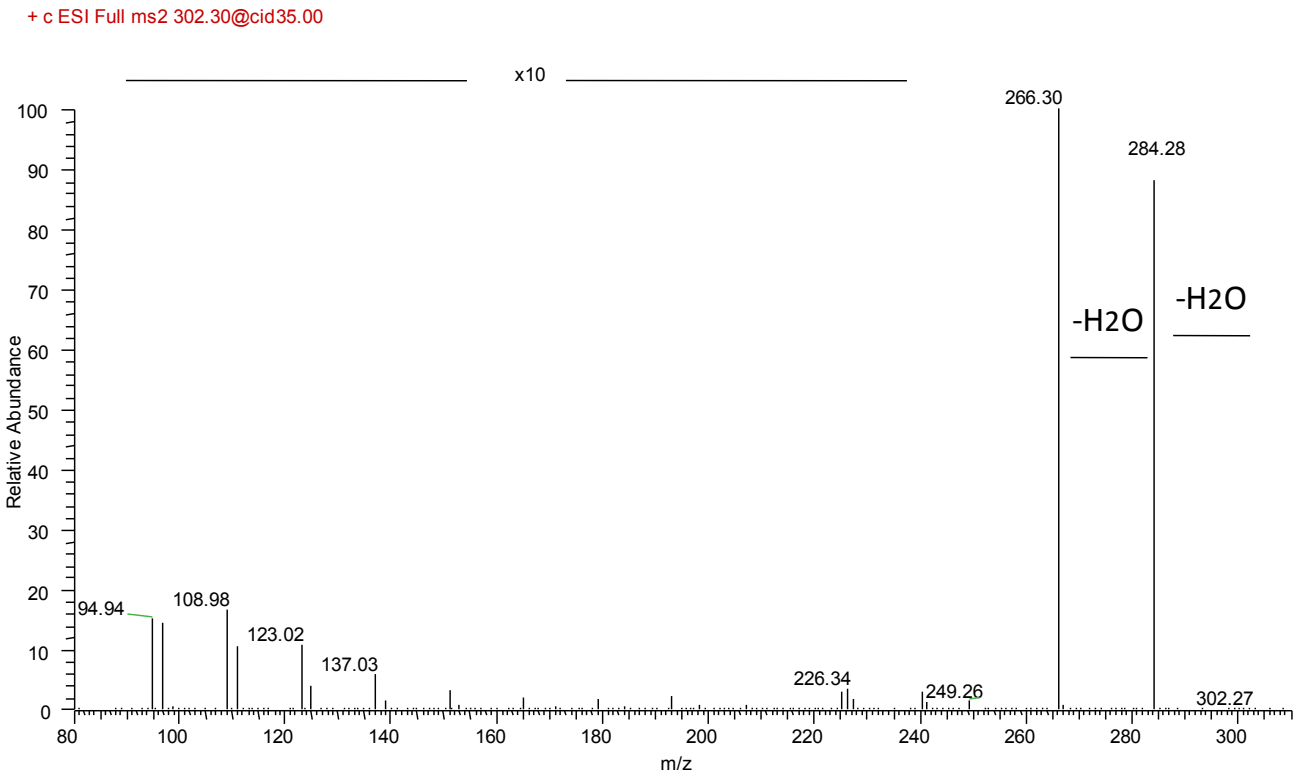

Figure S6. MS^2 spectrum of compound **4**

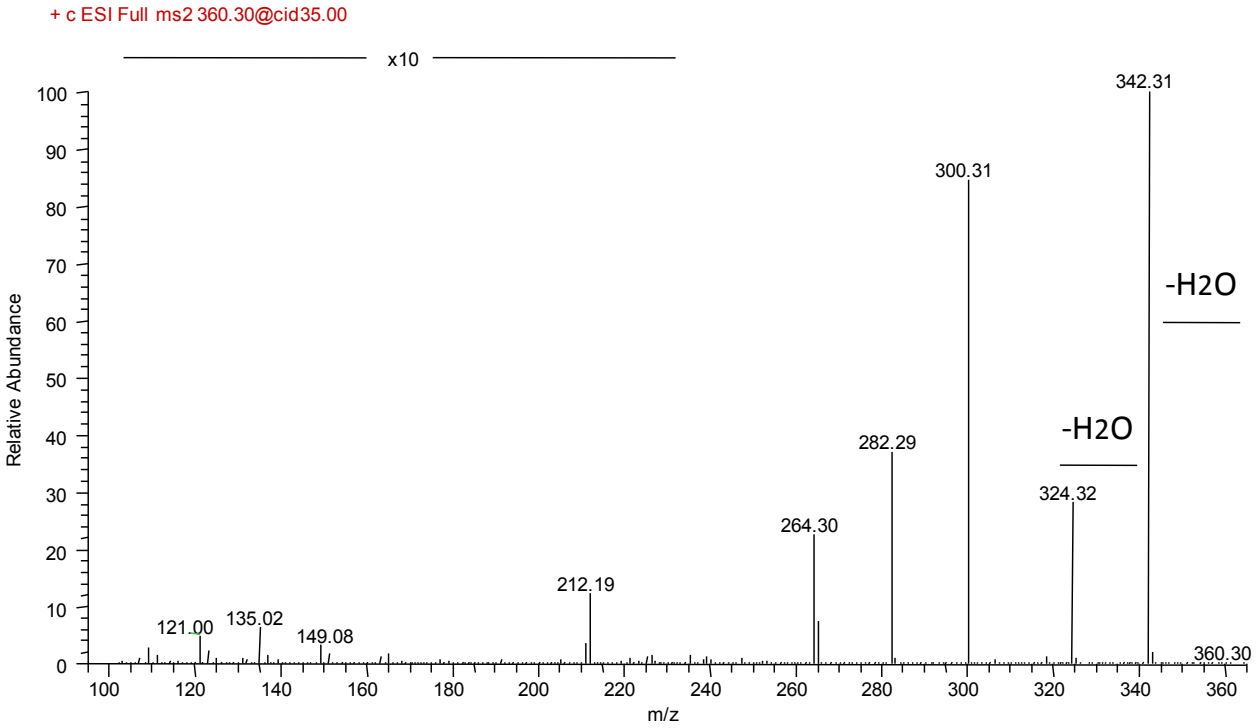

Figure S7. MS^2 spectrum of compound **6**

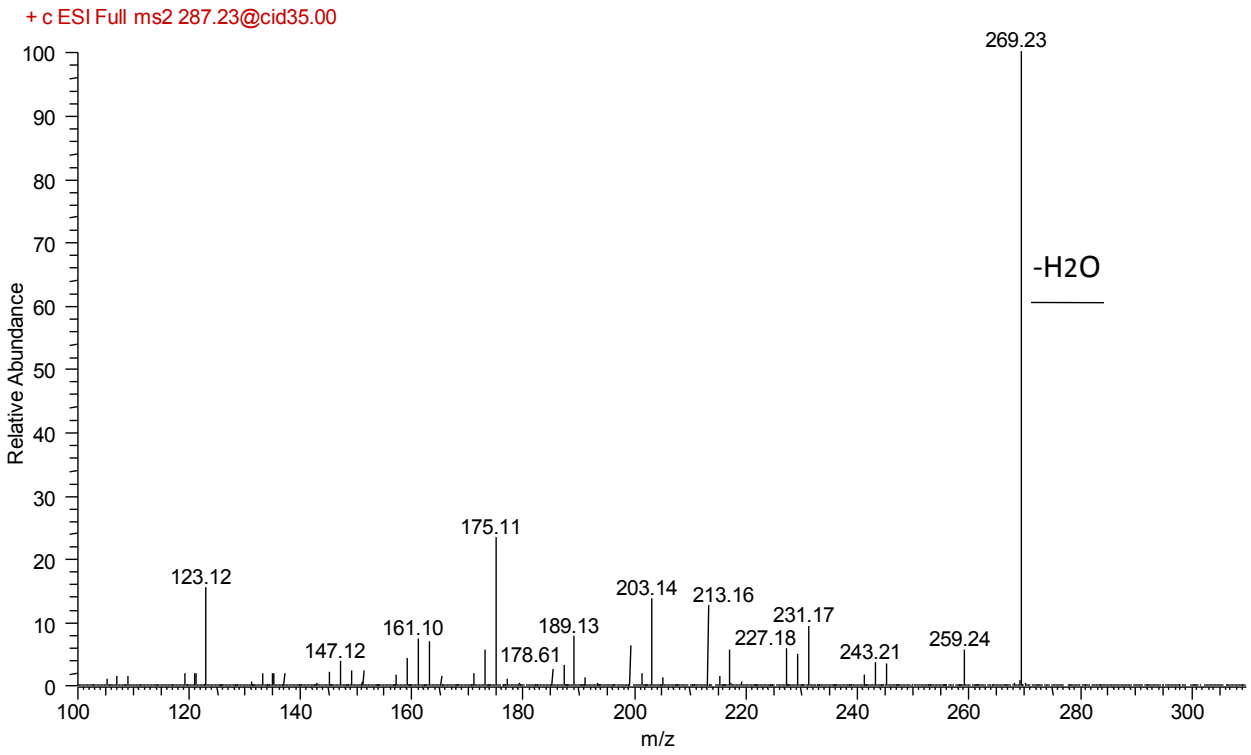

Figure S8. MS^2 spectrum of compound **7**

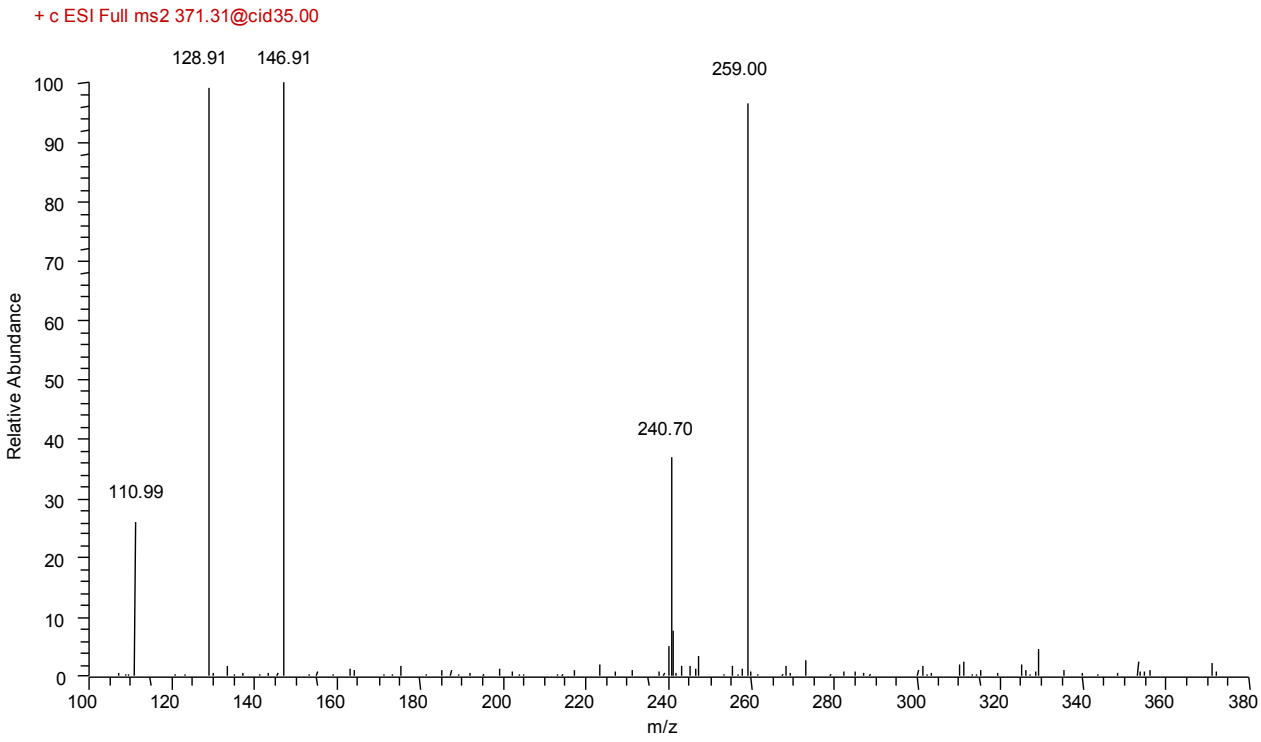

Figure S9. MS^2 spectrum of compound **8**

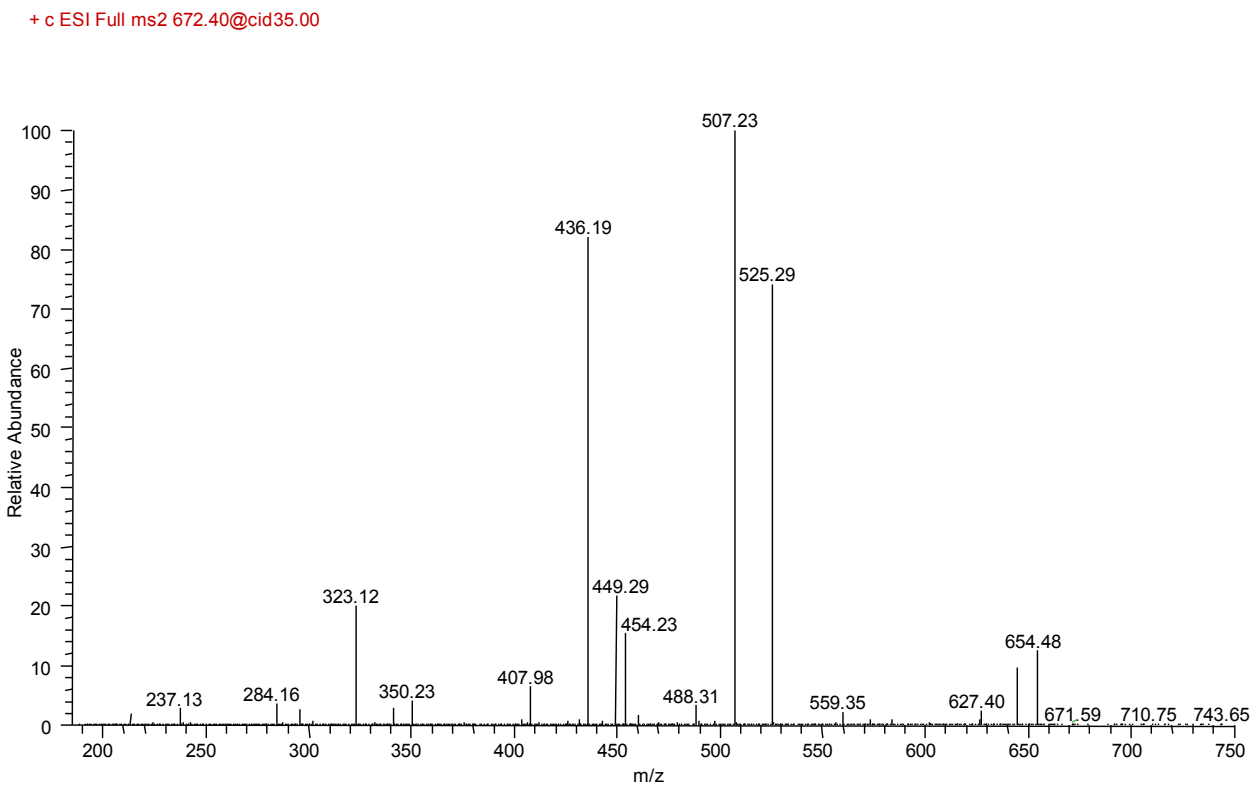

Figure S10. MS^2 spectrum of compound with molecular formula $C_{19}H_{39}NO_3$

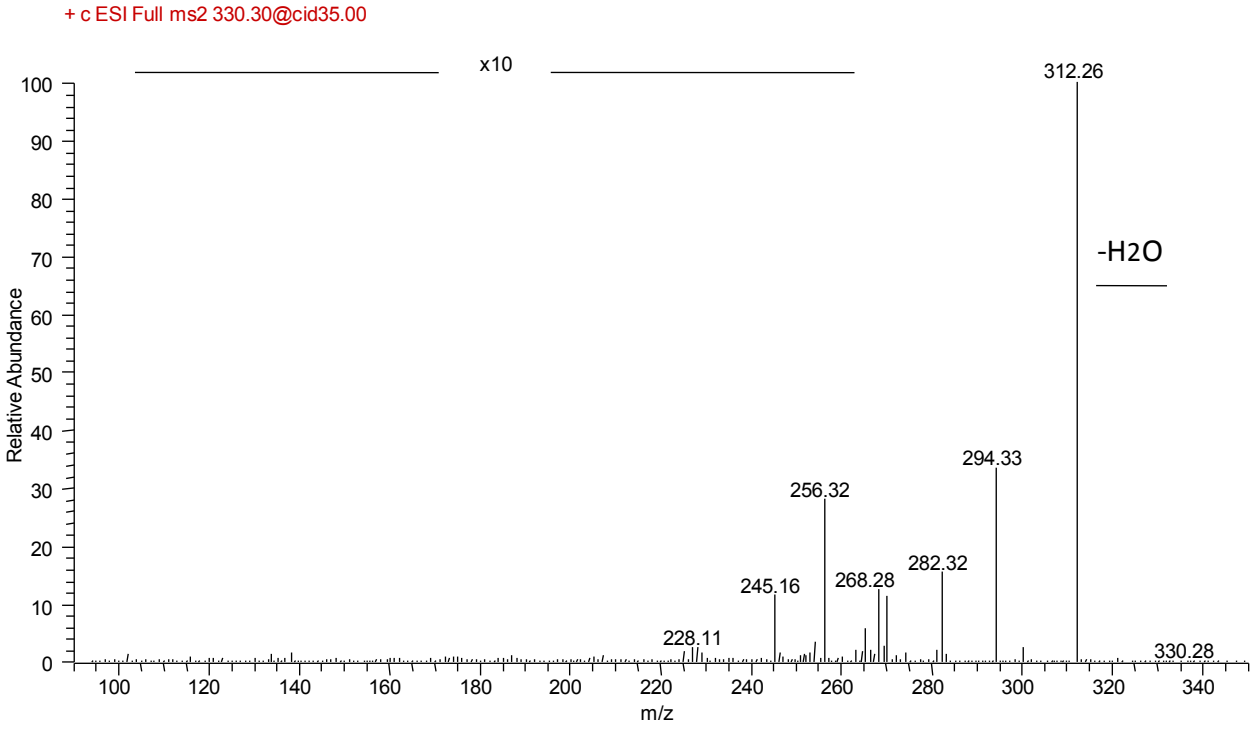

Figure S11. MS^3 data on the daughter ion of *m/z* 330.30