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1 **The antimicrobial potential of algicolous marine fungi for**
2 **counteracting multidrug resistant bacteria: phylogenetic diversity and**
3 **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 (IC₅₀), all crude extracts were subjected to preliminary ¹H 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.

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Keywords

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

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52 **Introduction**

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

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

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

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

75 **Materials and Methods**

76 **Fungal strains**

77 Fungi were isolated and roughly identified from the green alga *F. petiolata* collected in March
78 2010 near to Elba Island in the Mediterranean Sea [12], and are preserved at the *Mycotheca*
79 *Universitatis Taurinensis* - MUT (DBIOS - University of Turin). All the selected fungi were
80 revealed to be sterile mycelia and were identified by molecular analysis (**Table 1**).

81 **Molecular, Bioinformatics and Phylogenetic analyses**

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

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

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

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

98 Alignments were generated using MEGA 5.10 [16] and manually refined.
99 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 ddH₂O), MeCl (20 g malt extract, 17 g NaCl in 1 L of ddH₂O) 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

125 manner. The first step consisted of homogenization with Ultra Turrax T25 (IKA-Werke,
126 Staufen, Germany). The homogenate was then washed twice with 20 mL of EtOAc to recover
127 the intracellular extract; in addition, to improve the fungal lysis, mycelia were treated with
128 liquid nitrogen (15 mL N₂/g mycelium). Samples were transferred into a pre-cooled mortar
129 and minced under liquid nitrogen with a pestle and washed twice with 20 mL of EtOAc. At
130 the last step, to completely destroy the membrane, all the mycelium was transferred and
131 processed in a Potter-Elvehjem homogenizer (Sigma-Aldrich, Saint Louis, MO) in the
132 presence of EtOAc. Subsequently, the powdered mycelium was transferred into a separator
133 funnel and mixed five times with two volumes of EtOAc. In order to increase the yield of
134 some extracts, mycelia were further soaked in acetone for 18 hours under agitation. The
135 whole EtOAc and acetone fractions were collected and dried-out by using a Rotavapor. Final
136 extracts were weighed, solubilized in DMSO (100%) at a final concentration of 100 mg/mL
137 and stored at -20°C.

138 **Antimicrobial assay**

139 The extracts produced as such were checked for the ability to inhibit the growth of a
140 selected panel of human pathogens. An IC₅₀ assay was used to evaluate the concentration of
141 the extracts at which bacterial target growth was inhibited by 50%. The following multidrug
142 resistant bacteria were used for the antimicrobial screening: *Burkholderia metallica* LMG
143 24068 [19], *Pseudomonas aeruginosa* PA01 [20], *Klebsiella pneumoniae* DF12SA [21] and
144 *Staphylococcus aureus* 6538P [22]. All bacteria were routinely grown at 37°C in Lysogeny
145 broth (5 g yeast extract, 10 g sodium chloride, 10 g tryptone in 1 L of ddH₂O), with the
146 exception of *S. aureus*, which was grown in Mueller Hinton Broth (Applichem, Darmstadt,
147 Germany).

148 Extracts were placed into each well of a 96-well microtiter plate at an initial
149 concentration of 2 mg/mL and serially 2-fold diluted using the appropriate medium. Wells

150 containing only DMSO (2% v/v) were used as a control to determine the effect of this solvent
151 on bacterial growth.

152 Cells were prepared as follows: a single colony of each pathogenic strain was used to
153 inoculate 3 mL of liquid medium in a sterile bacteriological tube. After 5-8 h of incubation,
154 growth was measured by monitoring the absorbance at 600 nm and about 40,000 colony-
155 forming units were dispensed into each well of the prepared plate. Plates were incubated at
156 37°C for 20 h and growth was measured using a VICTOR X Multilabel Plate Reader
157 (PerkinElmer, Waltham, MA) by monitoring the absorbance at 600 nm.

158 **Metabolic profiling of crude extracts**

159 All crude extracts were subjected to Thin Layer Chromatography (TLC) analysis and
160 ¹H Nuclear Magnetic Resonance (NMR). TLC analysis was carried out on Alugram silica gel
161 G/UV254 plates with solvent mixture of different polarity using vanillin reagent as revelation
162 system; ¹H NMR analysis were performed with Varian INOVA 400 MHz instrument, in
163 CDCl₃ solvent, at room temperature with tetramethylsilane (TMS) as internal reference.

164 Selected extracts were analyzed using a LTQ XL Liquid Chromatography-High Resolution
165 Mass Spectrometry system (LC-HRMS) (ThermoScientific) equipped with the Accelera 600
166 Pump and Accelera Auto Sampler system. A volume of 10 µl of sample was injected at a
167 concentration of 10 mg/mL in methanol. The mixture was separated on a Phenomenex LUNA
168 C8 (150 X 2.1 mm, 5 µm particle size) column at a flow rate of 200 µL/min, using an
169 acetonitrile-water gradient. Mobile phase A was 90% H₂O 10% acetonitrile (ACN) 0.1%
170 formic acid (FA) and mobile phase B was 10% H₂O 90% ACN 0.1% FA; the gradient started
171 at 10% B up to 90% B in 70 min, was kept at 90% of B for 10 min before the re-equilibration
172 step. The mass spectrometer operated in positive electrospray ionization (ESI) mode, at 4 kV
173 capillary voltage and 280°C. The calibration procedure was carried out using
174 ThermoScientific positive calibration solution composed of caffeine, MRFA and Ultramark.

175 All spectra were acquired in the m/z range from 280 to 700 u.m.a., setting resolution at
176 30,000; MSMS spectra were acquired in an opportune m/z range using 35 of collision energy.
177 Thermo Scientific software Xcalibur was used to obtain molecular formulas. The Molecular
178 Formulas (MF) deduced by High-Resolution Electrospray Ionization Mass Spectrometry
179 (HRESIMS) were checked by available data banks [23-25] and, in the case of alternative
180 structures, they were discriminated by MSⁿ analysis using the data available in the literature
181 [26] or *ex-novo* analysis, and then by checking diagnostic signals in the ¹H NMR spectrum of
182 the crude extracts.

183 **Results**

184 **Phylogeny and taxonomic identification of the fungal isolates**

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

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

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

197 **Antimicrobial activity**

198 In order to select the best growth medium for producing the antimicrobial compounds,
199 preliminary extractions and antimicrobial assays were performed on small-scale cultures of

200 fungi grown in MeCl, PCB and WST30. These analyses demonstrated that fungi grown in
201 MeCl exhibited the highest degree of antimicrobial activity (Supplementary materials **Table**
202 **S1**). This medium was therefore selected for further experiments. Moreover, the antimicrobial
203 potentials of the extracellular and intracellular extracts were compared; results revealed that
204 the latter exhibited the highest yield and activity (Supplementary materials **Table S2**).

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

211 Extracts produced from strains MUT 4861, MUT 4865, and MUT 4979 resulted as
212 being the most active and promising ones. In particular, MUT 4861 was able to strongly
213 inhibit *B. metallica* (IC_{50} 0.5-0.25 mg/mL) and *S. aureus*, and was the only one to show, by
214 both EtOAc and acetone extracts, an inhibitory effect against *P. aeruginosa*. Both extracts
215 from MUT 4865 were able to inhibit *B. metallica* and *S. aureus* (IC_{50} 0.5-0.25) and the
216 EtOAc extracts also showed inhibition against *K. pneumoniae*. No effects were observed
217 against *P. aeruginosa*. The extract from MUT 4979 showed antimicrobial activity against
218 three out of the four pathogens (IC_{50} 1.0-0.25), with the exception of *K. pneumoniae*. Extracts
219 of MUT 4859, 4860, and 4966 only showed a significant activity against *B. metallica* and *S.*
220 *aureus*, which were the most sensitive bacterial strains to the fungal extracts. MUT 4883,
221 4885 and 4886 extracts were the weakest strains showing no significant effects against the
222 target bacteria. Acetone extracts showed similar antimicrobial activity compared to EtOAc
223 extracts. The only exception was MUT 4861, of which the acetone extract was more active
224 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 C₂₂H₄₃O₂N by HRMS analysis
235 (calculated for C₂₂H₄₃NO₂Na: 376.3192, found [M + Na]⁺: 376.3195). In the MS² 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 MS³ 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 MS² pattern analysis (**Table 3** and
248 Supplementary materials **Fig. S4-S6**) leads to a straightforward assignment of a planar
249 structure to these compounds.

250 Compound **5**, which was present in both EtOAc and acetone extracts, was tentatively
251 identified as aphidicolin; compound **6** was tentatively identified as fusoxysporone and
252 compound **7**, a minor component of the EtOAc extract, was identified as bis (2-ethylhexyl)
253 hexanedioic acid.

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

259 In addition to phytosphingosine (**2**), an "unusual" sphingoid base with a molecular
260 formula C₁₉H₃₉NO₃ was detected. The MS² spectrum (Supplementary materials **Fig. S10**)
261 showed fragmentation peaks resulting in the sequential loss of three water molecules, whereas
262 no ammonia elimination was measured. This finding could suggest the involvement of a
263 nitrogen atom in an azetidine ring, as in isomeric penaresidins A and B.

264 Although the fragmentation pattern observed in the MS³ spectrum (Supplementary
265 materials **Fig. S11**) is compatible with these structures, no ambiguous information relative to
266 the position of the hydroxyl groups, of the methyl branching, or even on the nature of
267 unsaturation, can be drawn.

268 Finally, Scopularide A (**8**) [28] was identified by MF analysis and by diagnostic MS²
269 fragmentations (**Table 4** and Supplementary materials **Fig. S9**).

270 **Discussion**

271 In this study, the green marine alga *F. petiolata* was chosen as a source of promising
272 marine fungi since it has been previously demonstrated that fungi isolated from marine algae
273 showed strong antimicrobial activity against several human pathogenic bacteria [29],
274 probably deriving from the ability to protect their algal host from external threats [30].

275 Identifying new fungal strains could lead to the discovery of new and unusual compounds,
276 which can be utilized for biotechnological and pharmaceutical applications.

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

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

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

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

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

297 As the antimicrobial activity of these algicolous fungi on MDR bacteria (according to
298 the results of the bioassay tests) were in agreement with the known antimicrobial potential of
299 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 *Penicillium* 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

325 pathological conditions [42]. In particular, a recent study [43] revealed that sphingoid long-
326 chain bases displayed antibacterial activity against a broad spectrum of pathogenic bacteria,
327 including *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Haemophilus influenzae*,
328 *Moraxella catarrhalis* and even *Burkholderia cepacia*, at nanomolar-to-low micromolar
329 concentrations. Therefore, even though we cannot exclude, *a priori*, the possibility that the
330 antimicrobial activity could rely on the combination of different molecules, compound **1**, and
331 co-occurring sphingosines **2**, **3** and **4**, previously reported as common components of fungal
332 membrane sphingolipids [44], may be responsible for the antimicrobial effects exhibited by
333 MUT 4865 crude extracts towards the pathogenic bacteria investigated so far. However, tests
334 with the purified compound will be necessary to validate this hypothesis.

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

341 Compound **7**, identified as bis (2-ethylhexyl) hexanedioic acid, is known as plasticizer
342 [48] and described as a component of cyanobacteria, Antarctic [49] and terrestrial [50] strains
343 of *Streptomyces*, and of a tropical plant [51].

344 Sphingosine-related compounds were also detected in the EtOAc extract of
345 *Microascacea* sp.2 MUT 4861, which also contains a member of the class of so-called
346 anhydrophytosphingosines, in particular the detected compound is isomeric with azetidine-
347 derived penaresidins A and B, which were first isolated from the marine sponge *Penares* sp.
348 [52].

349 Conversely, compound **8** is a cyclodepsipeptide scopularide A, a molecule with

350 antiproliferative activity, previously isolated from a marine strain of the fungus
351 *Scopulariopsis brevicaulis* [28], belonging to the same Microascaceae family assigned to
352 MUT 4861.

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

366

367 **Conflicts of Interest**

368 The authors declare that there are no conflicts of interest.

369

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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**.

526

527 **Fig. 2.** MS³ 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 accession numbers.

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MUT Code	Fungal taxa	GenBank accession number ITS and LSU
4883	<i>Biatriospora</i> sp.	KR014352 KP671728
4865	<i>Beauveria bassiana</i> (Bals.-Criv.) Vuill.	KR014380 KP671729
4860	<i>Massarina</i> sp.	KR014362 KP671730
4885	Microascacea sp. 1	KR014356 KP671717
4861	Microascacea sp. 2	KR014360 KP671746
4859	Rousoellacea sp. 1	KR014355 KP671716
4886	Rousoellacea sp. 2	KR014358 KP671720
4966	Rousoellacea sp. 3	KR014366 KP671740
4979	<i>Knufia petricola</i> (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% (IC₅₀). 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)							
	<i>B. metallica</i> LMG 24068		<i>P. aeruginosa</i> PA01		<i>K. Pneumoniae</i> DF12SA		<i>S. aureus</i> 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] ⁺ (Δ ppm: 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 (Δ ppm: -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 (Δ ppm: 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 (Δ ppm: -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 (Δ ppm: - 0.876)	C ₂₀ H ₃₄ O ₄	Aphidicolin (5)
54.04	287.23634 (Δ ppm: 0.584) MS ² (Fig. S7): 269.23, 203.14, 175.11	C ₂₀ H ₃₀ O	Fusoxysporone (6)
60.38	395.3309 (Δ ppm: 0.145)	C ₂₈ H ₄₂ O	Ergosta-5,7,22-trien-3- β -ol (ergosterol)
62.89	393.3153 (Δ ppm: 0.401)	C ₂₈ H ₄₀ O	Ergostane derivative
66.49	371.31453 (Δ ppm: -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 (Δ ppm - 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 (Δ ppm - 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 (Δ ppm- 0.166) MS ² (Fig. S9) 654.5, 525.3, 507.2, 454.2, 436.2, 323.1	C ₃₆ H ₅₇ N ₅ O ₇	Scopularide A (8)
58.14	409.3101 (Δ ppm 0)	C ₂₈ H ₄₀ O ₂	Ergostane derivative
59.94	393.3154 (Δ ppm 0)	C ₂₈ H ₄₀ O	Ergostane derivative
65.6	395.3307 (Δ ppm 0)	C ₂₈ H ₄₂ O	Ergosterol
73.06	371.31576 (Δ ppm 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 (Δ ppm 0)	C ₂₈ H ₄₀	Ergosta-3,5,7,9(11),22-pentaene

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Supporting Information

616 **The antimicrobial potential of algicolous marine fungi for counteracting** 617 **multidrug resistant bacteria: phylogenetic diversity and chemical profiling**

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

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621 **Legend to Supplementary figures**

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

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

634 **Fig. S3.** MS² spectrum of compound 1.

635 **Fig. S4.** MS² spectrum of compound 2.

636 **Fig. S5.** MS² spectrum of compound 3.

637 **Fig. S6.** MS² spectrum of compound 4.

638 **Fig. S7.** MS² spectrum of compound 6.

639 **Fig. S8.** MS² spectrum of compound 7.

640 **Fig. S9.** MS² spectrum of compound 8.

641 **Fig. S10.** MS² spectrum of compound with molecular formula C₁₉H₃₉NO₃

642 **Fig. S11.** MS³ data of compound with molecular formula C₁₉H₃₉NO₃ on the daughter ions of
643 *m/z* 330.30.
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647 **Table S1. Selection of the best fungi growth media antimicrobial compounds production.**
 648 Table reports the antimicrobial activity as the percentage of inhibition of a selected target
 649 bacterium (*Burkholderia metallica* LMG 24068) in presence of the fungal extracellular
 650 extracts from the three different growth media. MeCl medium showed the best antimicrobial
 651 activity. ND: Not detected.

MUT Code	Growth media		
	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

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

MUT Code	Intracellular extract	Extracellular extract	
4859	70 ± 3.4	40 ± 3.2	693
4860	67 ± 2.1	33 ± 1.3	694
4861	56 ± 0.9	30 ± 0.5	695
4865	60 ± 2.5	32 ± 0.7	696
4883	54 ± 3.1	25 ± 0.8	698
4885	76 ± 4.3	33 ± 1.2	700
4886	60 ± 3.8	10 ± 0.6	702
4966	60 ± 2.1	15 ± 1.3	
4979	60 ± 6.5	30 ± 2.1	704

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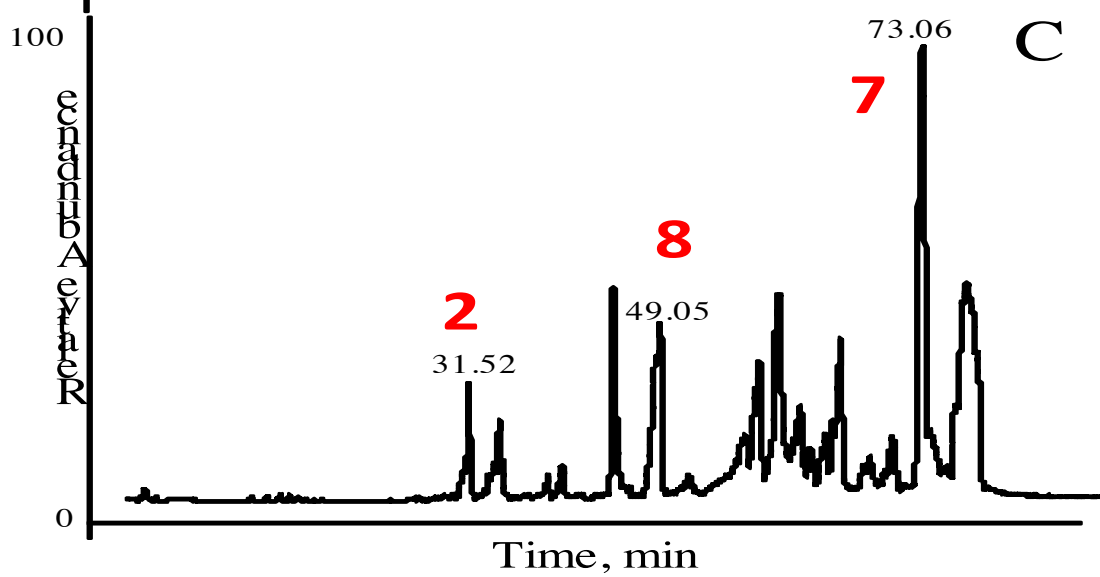
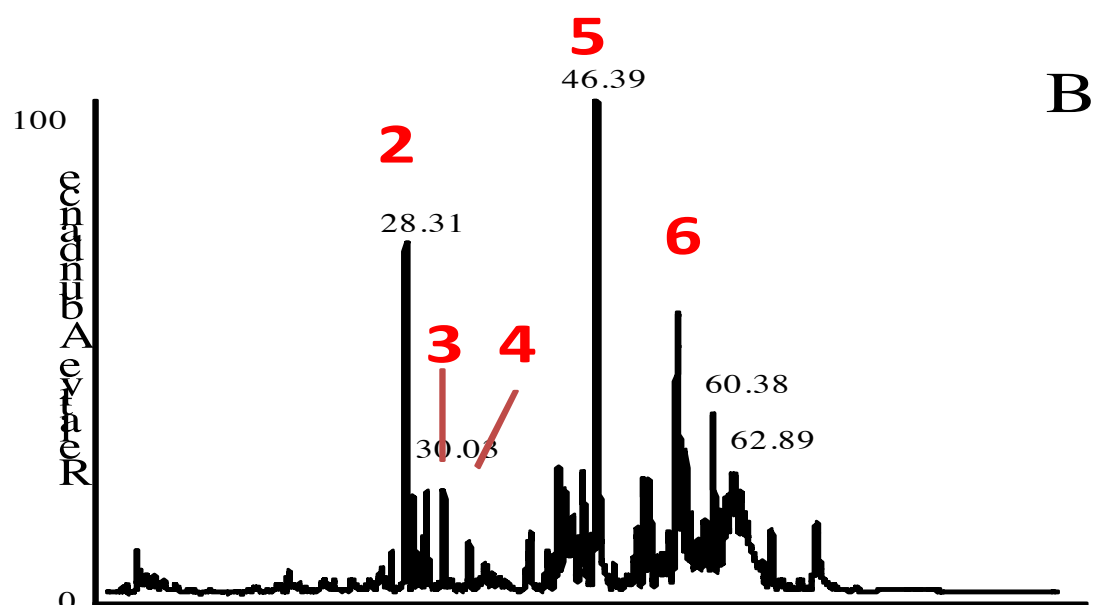
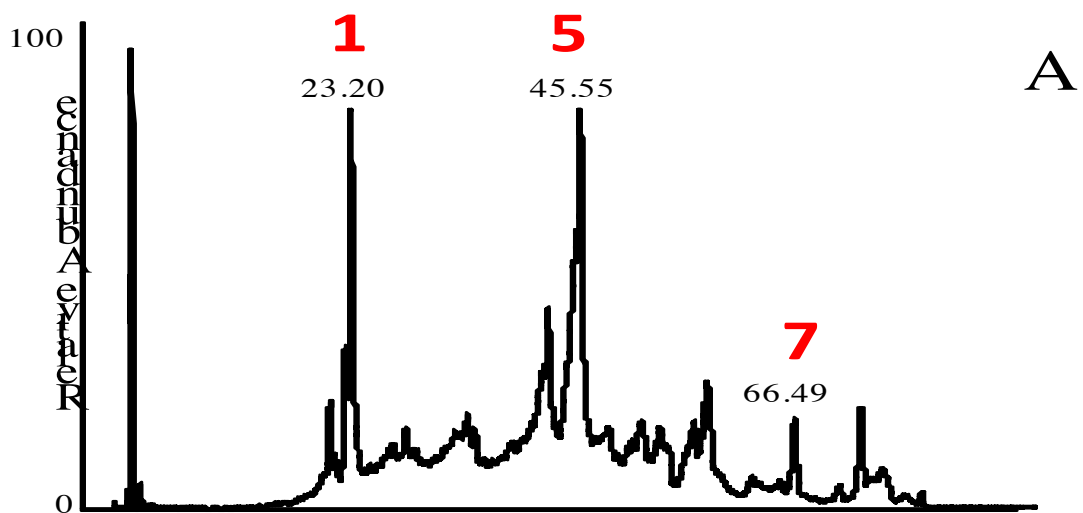
725 **Table S3. Re-assessment of the antibiotic resistance of the four MDR bacterial strains**
 726 **belonging to different species.** The data are reported as capacity to inhibit the
 727 microorganism growth in more than 50% (IC₅₀).

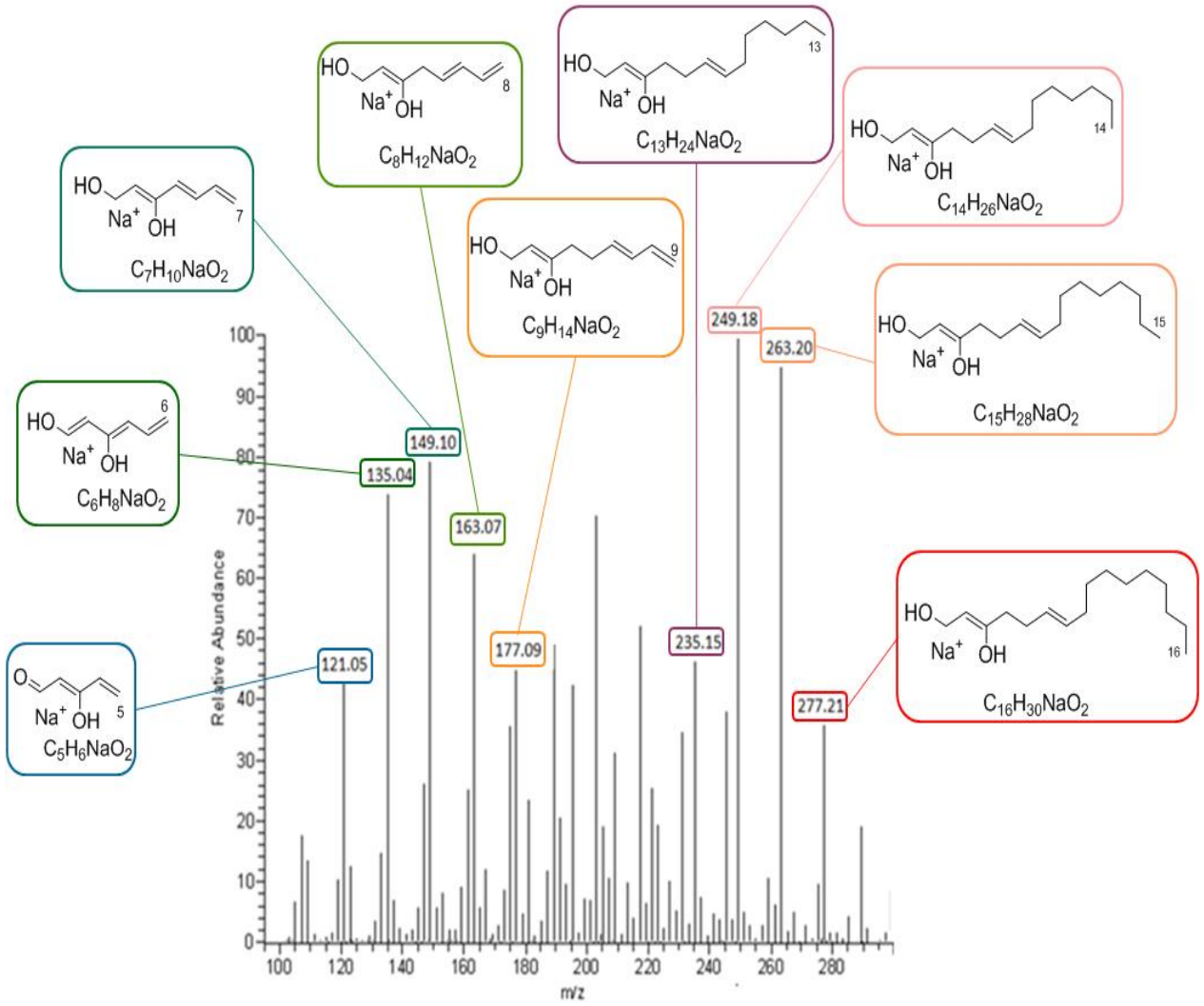
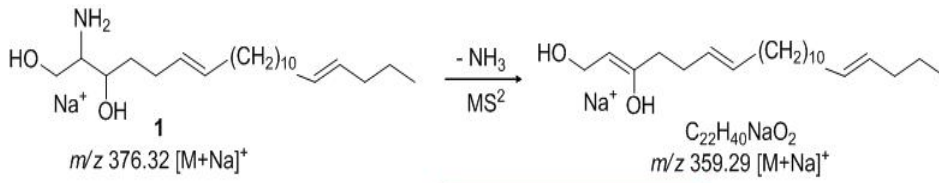
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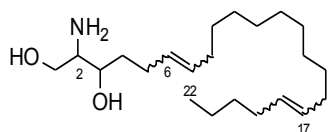
Antibiotic	IC ₅₀ (mg/mL)			
	<i>B. metallica</i> LMG 24068	<i>P. aeruginosa</i> PA01	<i>K. pneumoniae</i> DF12SA	<i>S. aureus</i> 6538P
Ampicillin	> 0.2	0.025 - 0.012	< 0.003	< 0.003
Chloramphenicol	0.006 - 0.003	0.006 - 0.003	< 0.003	< 0.003
Kanamycin	0.006 - 0.003	0.006 - 0.003	< 0.003	< 0.003
Tetracycline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

729

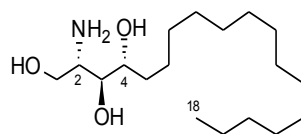
730



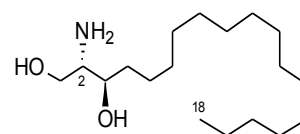




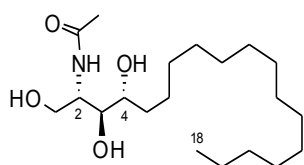
1,3-dihydroxy-2-amino-6,17-docosadiene (1)



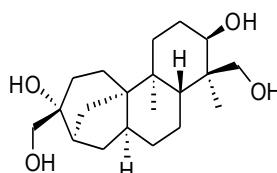
1,3,4-trihydroxy-2-aminooctadecane (2)



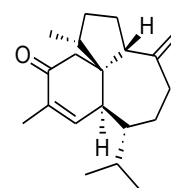
1,3-dihydroxy-2-aminooctadecane (3)



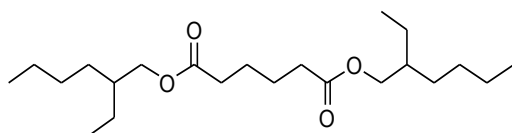
N-Acetyl-1,3,4-trihydroxy-2-aminooctadecane (4)



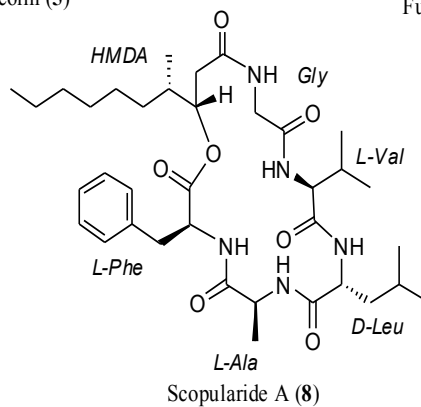
Aphidicolin (5)



Fusoxysporone (6)

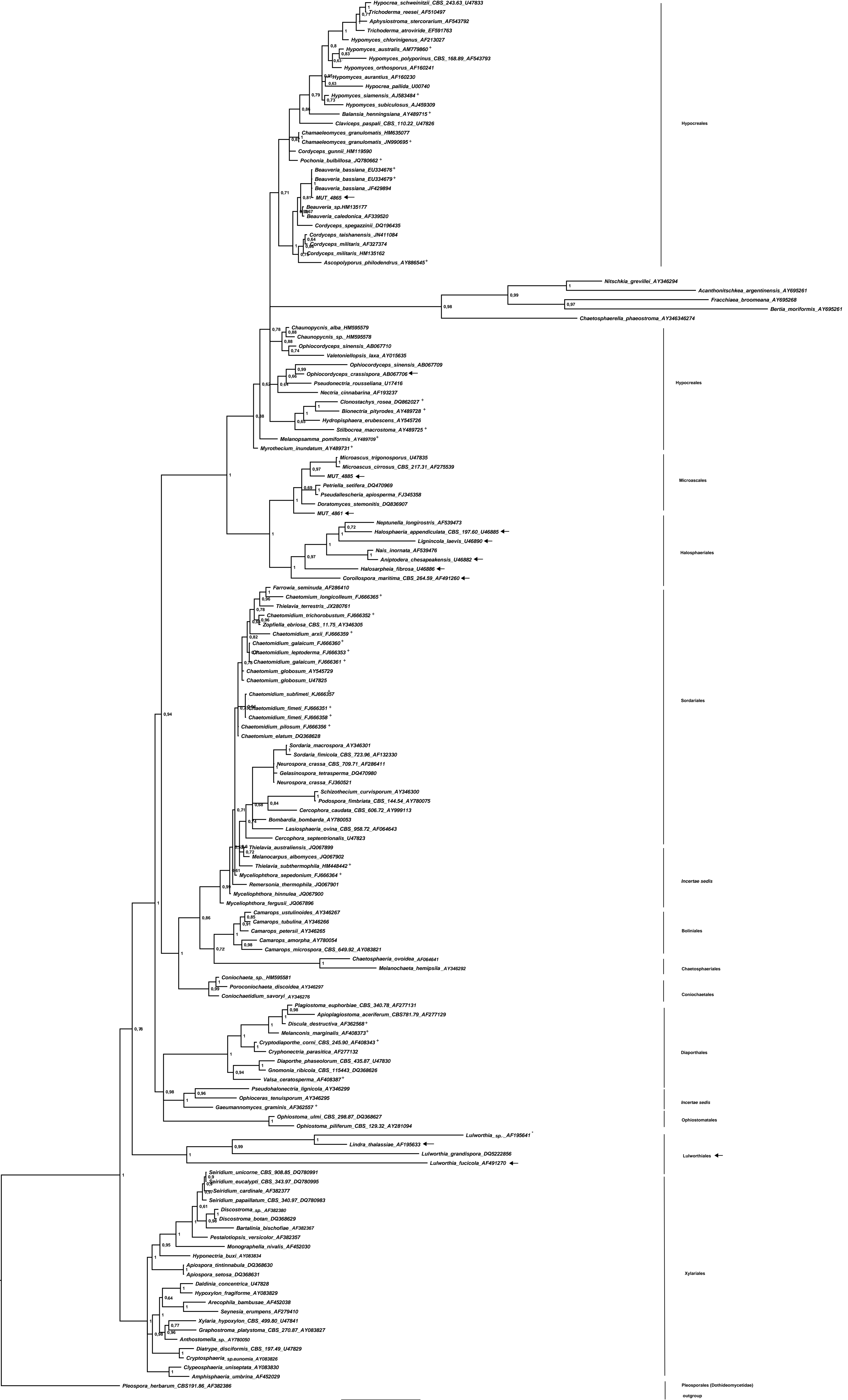


Bis-(2-ethylhexyl)-hexanedioic acid (7)



Scopularide A (8)





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Figure S3. MS² spectrum of compound 1

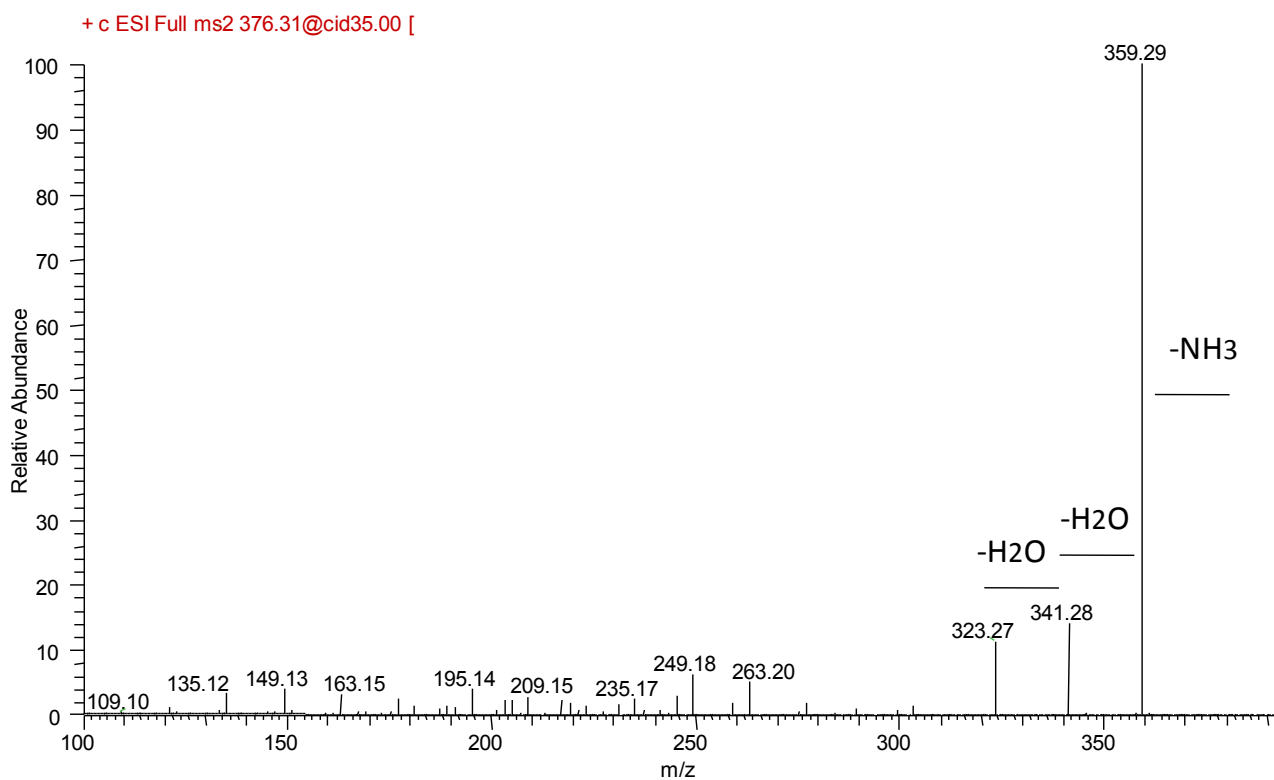


Figure S4. MS² spectrum of compound 2

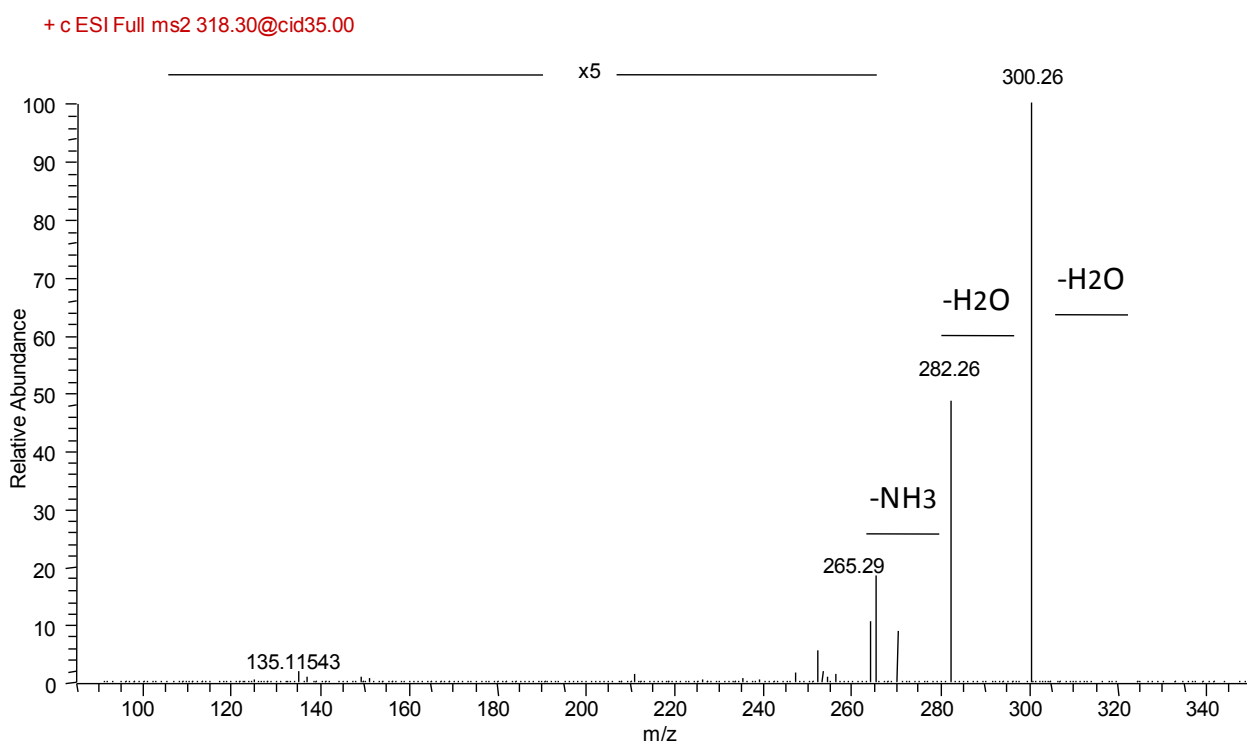


Figure S5. MS² spectrum of compound **3**

+ c ESI Full ms2 302.30@cid35.00

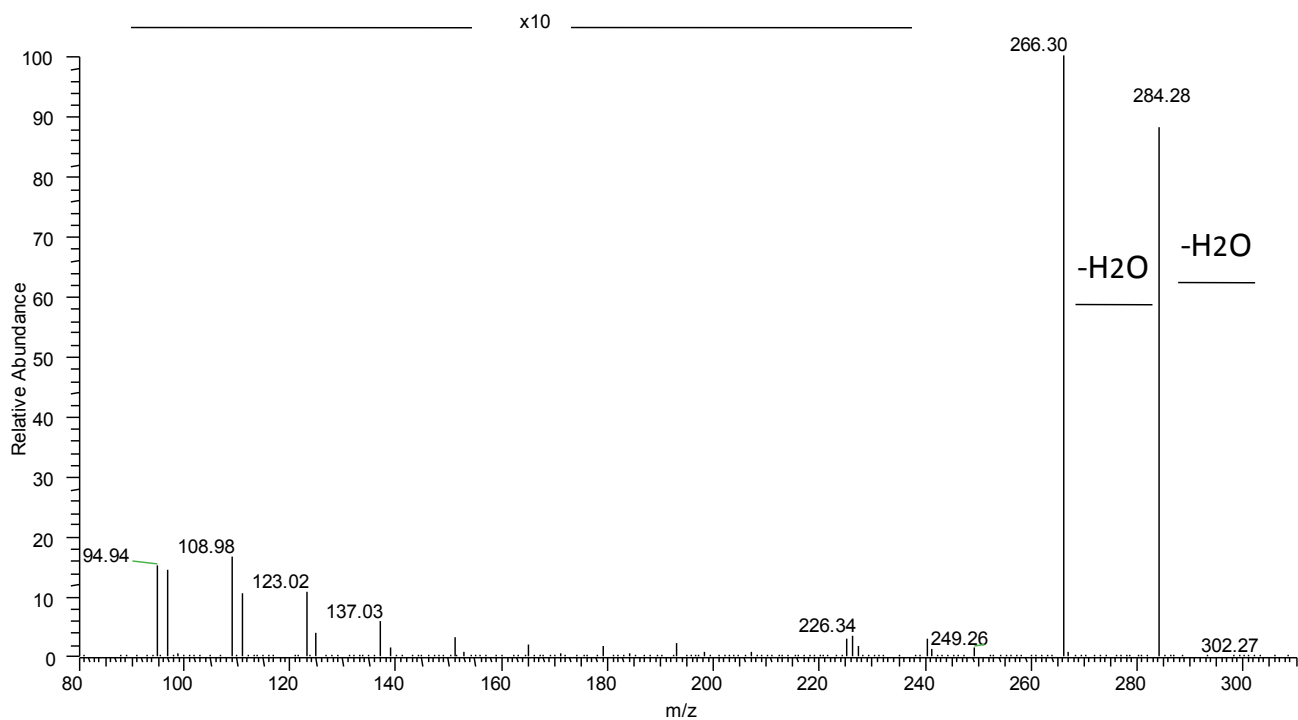


Figure S6. MS² spectrum of compound **4**

+ c ESI Full ms2 360.30@cid35.00

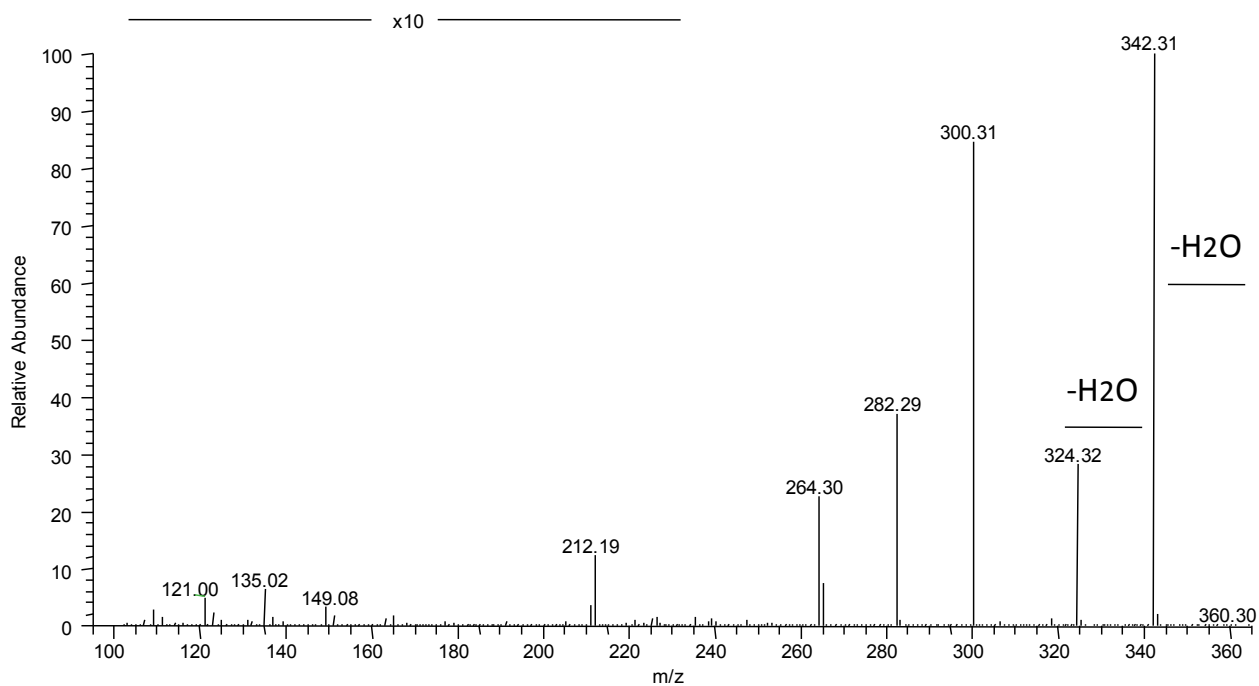


Figure S7. MS² spectrum of compound 6

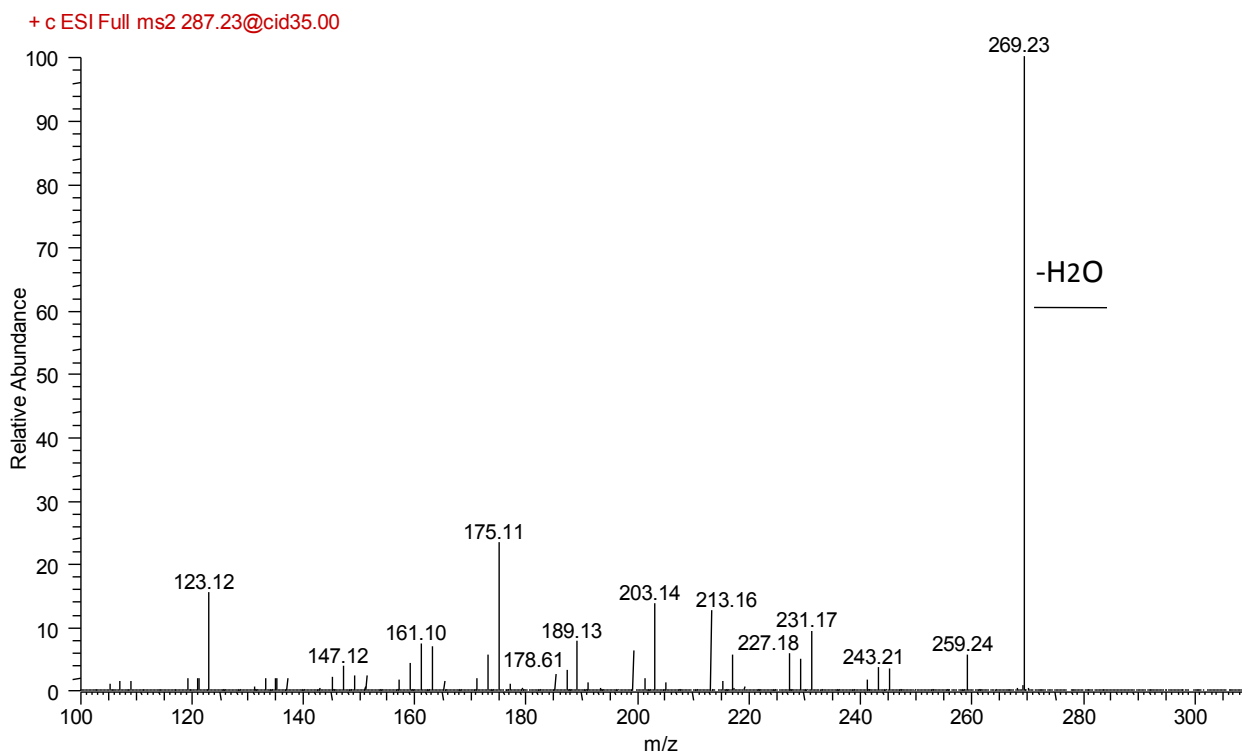


Figure S8. MS² spectrum of compound 7

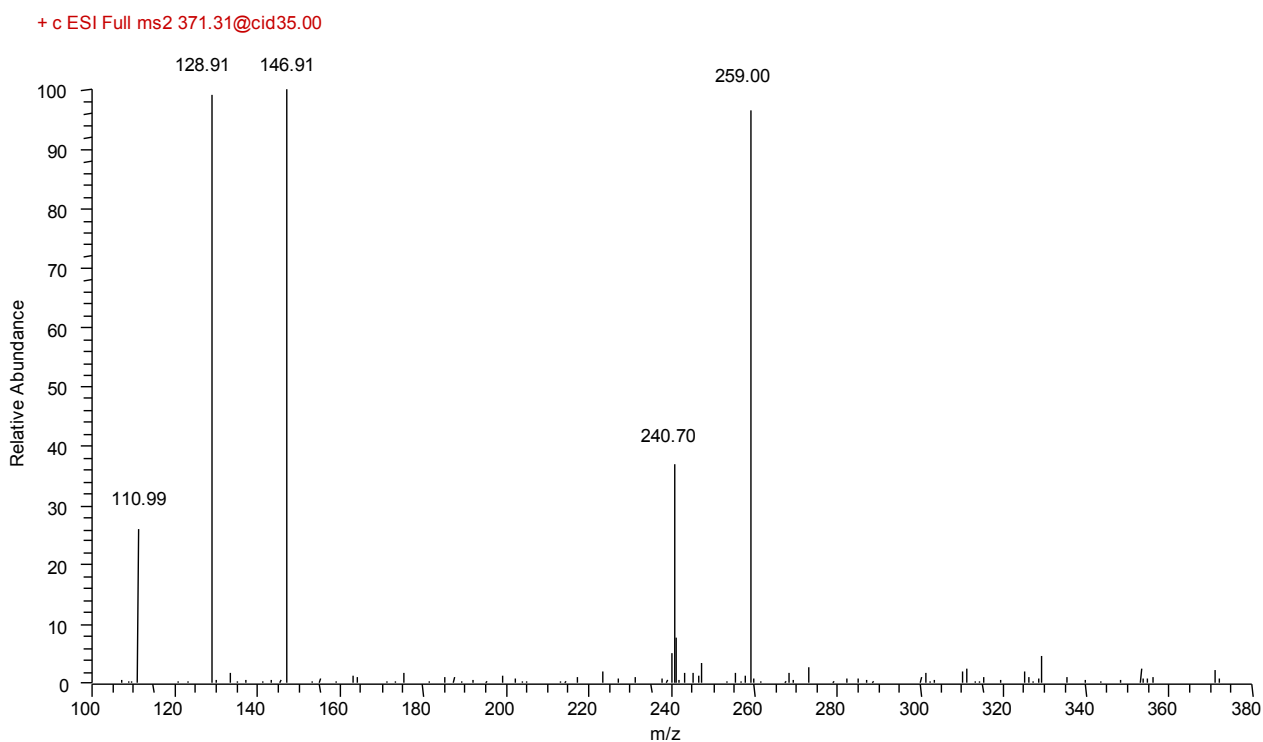


Figure S9. MS² spectrum of compound **8**

+ c ESI Full ms2 672.40@cid35.00

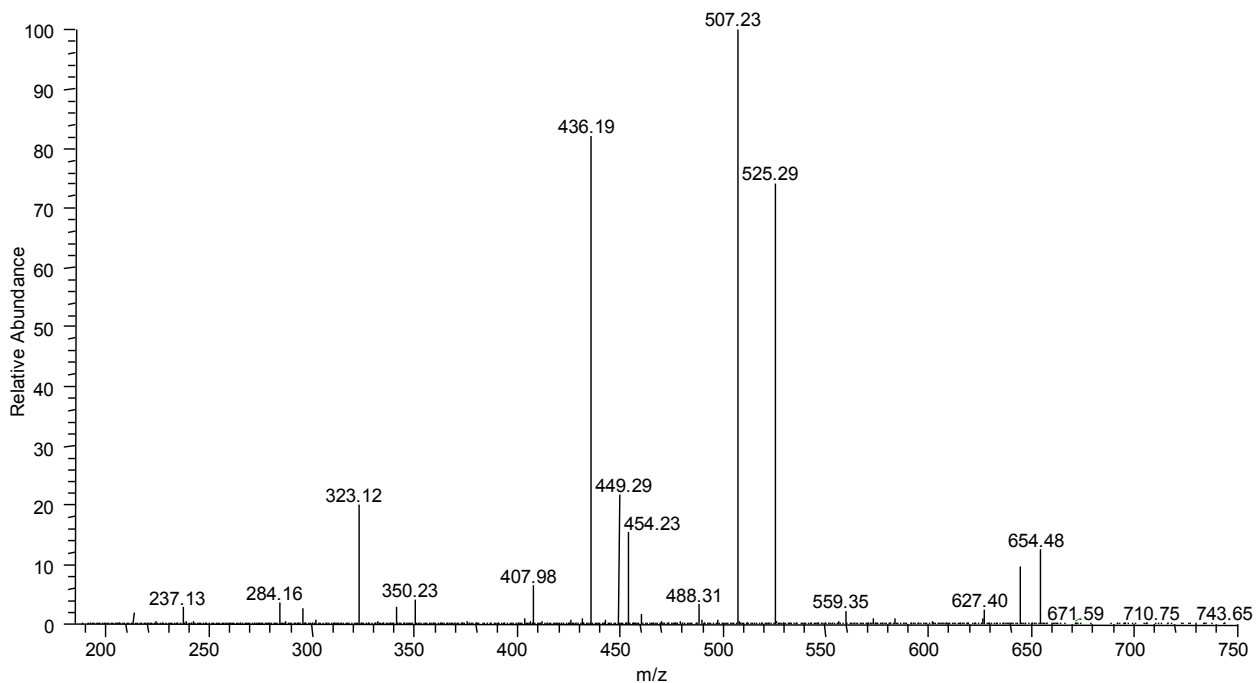


Figure S10. MS² spectrum of compound with molecular formula C₁₉H₃₉NO₃

+ c ESI Full ms2 330.30@cid35.00

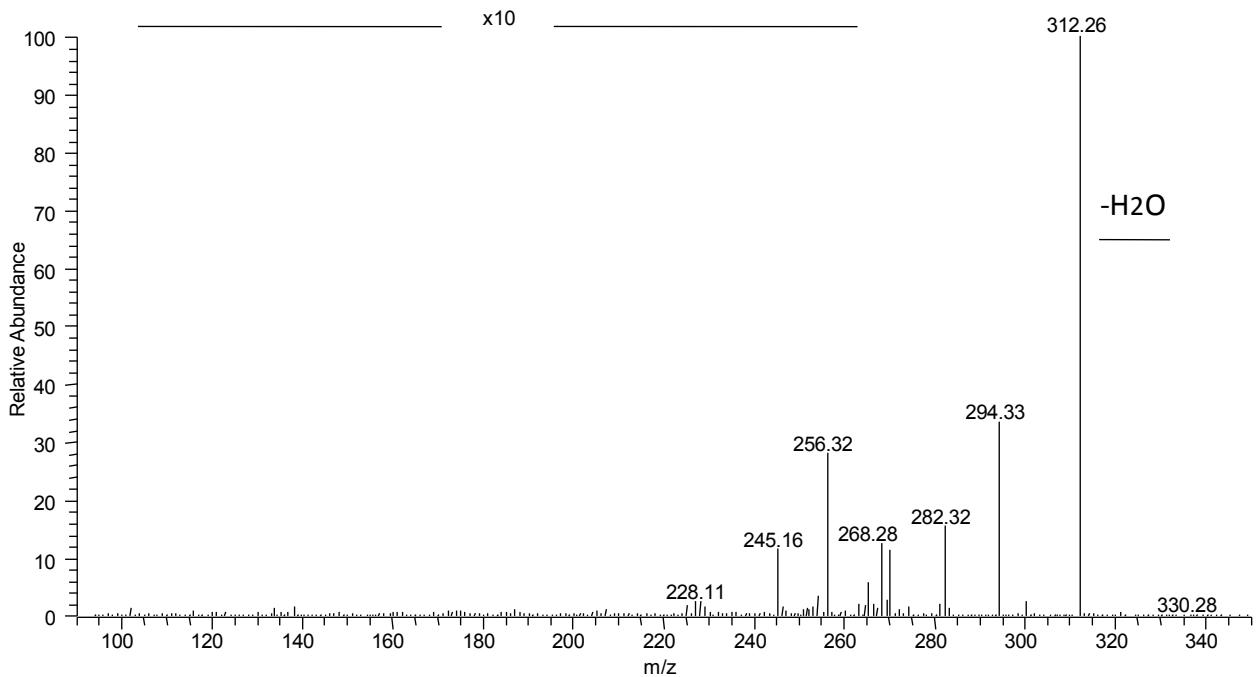


Figure S11. MS³ data on the daughter ion of m/z 330.30

+ c ESI Full ms3 330.30@cid35.00 294.30@cid35.00

