Bioactive secondary metabolites produced by the emerging pathogen Diplodia olivarum

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Summary. A new cleistanthane nor-diterpenoid, named olicleistanone (1), was isolated as a racemate from the culture filtrates of Diplodia olivarum, an emerging pathogen involved in the aetiology of branch canker and dieback of several plant species typical of the Mediterranean maquis in Sardinia, Italy. When the fungus was grown in vitro on Czapek medium, olicleistanone was isolated together with some already known phytotoxic diterpenoids identified as sphaeropsidins A, C, and G, and diplopimarane (2-5). Olicleistanone was characterized as 4-ethoxy-6a-methoxy-3,8,8-trimethyl-4,5,8,9,10,11-hexahydrodibenzo[de,g]chromen-7(6aH)-one. When D. olivarum was grown on mineral salt medium it produced (-)-mellein (6), sphaeropsin A and small amounts of sphaeropsidin G and diplopimarane. Olicleistanone (1) exhibited strong activity against the insect Artemia salina L. (100% larval mortality) at 100 μg mL⁻¹ but did not exhibit phytotoxic, antifungal or antioomycete activity. Among the metabolites isolated (1-6), sphaeropsidin A (2) was active in all bioassays performed exhibiting strong phytotoxicity on leaves of Phaseolus vulgaris L., Juglans regia L. and Quercus suber L. at 1 mg mL⁻¹. Sphaeropsidin A (2) also completely inhibited mycelium growth of Athelia rolfsii, Diplodia corticola, Phytophthora cambivora and P. lacustris at 200 μg per plug, and was active in the Artemia salina assay. Also in this assay, diplopimarane (5) and sphaeropsidin G (4) were active (100% larval mortality). Diplopimarane also showed antifungal and antioomycete activities. Athelia rolfsii was the most sensitive species to diplopimarane. Sphaeropsidin C (3) and (-)-mellein (6) were inactive in all bioassays. These results expand knowledge on the metabolic profile of Botryosphaeriaceae, and embody the first characterization of the main secondary metabolites secreted by D. olivarum.

Keywords. Botryosphaeriaceae, forest ecosystems, olicleistanone, toxins.

INTRODUCTION

Diplodia Fr. is a large genus in the Botryosphaeriaceae, typified by Diplodia mutila (Fr.: Fr.) Fr. (Alves et al., 2014). Species of Diplodia are cosmopolit-
tan in temperate and subtropical regions, and occur on a wide range of angiosperm and gymnosperm hosts (Masi et al., 2018). They exhibit diverse lifestyles, from endophytes inhabiting asymptomatic plant tissues to aggressive pathogens that cause severe diseases in various plant hosts (Pérez et al., 2010; Adamson et al., 2015; Martin et al., 2017; Masi et al., 2018). The increasing number of reports of new diseases caused by these pathogens has stimulated research into the virulence factors involved in their pathogenesis processes. Several bioactive secondary metabolites were isolated and characterized from the emerging Diplodia pathogenic species D. africana, D. corticola, D. cupressi, D. fraxini, D. quercivora and D. sapinea. These metabolites belong to different classes of organic compounds including pimarane diterpenoids, α-pyrones, furanones, diplobifuranylones, naphthoquinones, biphenols, cyclohexene oxides, furopyrans, isochromanones and melleins (Evidente et al., 2012; Andolfi et al., 2014; Cimmino et al., 2016; Cimmino et al., 2017a; Masi et al., 2018). Some phytotoxins produced by Diplodia species (e.g. the tetracyclic pimarane diterpenoid, sphaeropsidin A) have broad-spectra of biological properties, including anticancer activity (Masi et al., 2018).

Recently, Diplodia olivarum has emerged as an aggressive pathogen on different plant hosts in Italy. This fungus was first found on rotting olive drupes in southern Italy, and was described as a new species in 2008 (Lazzizera et al., 2008). It was later reported as a cause of canker on carob tree (Granata et al., 2011), lentisk (Linaldeddu et al., 2016) and wild olive (Manca et al., 2020). Symptoms caused by the pathogen in infected hosts include sunken cankers with characteristic wedge-shaped wood necroses on branches and stems. Foliar symptoms have also been observed especially on lentisk shoots (Figure 1).

Given the expansion of severe dieback caused by D. olivarum in several natural ecosystems in Italy, and the limited information available about bioactive secondary metabolites produced by this emerging pathogen, the study described here was conducted to isolate, identify and evaluate phytotoxic, antifungal, antioomycetes and zootoxic activities of the main compounds produced by D. olivarum.

**MATERIALS AND METHODS**

*Chemical characterization procedures*

Optical rotations were measured in MeOH on a P-1010 digital polarimeter (Jasco, Tokyo, Japan), unless otherwise noted. IR spectra were recorded as a glass film deposits using a 5700 FT-IR spectrometer (Jasco), and UV spectra were measured in MeCN on a V-530 spectrophotometer (Easton). 1H and 13C NMR spectra were recorded, respectively, at 400 and 100 MHz in CDCl3, on a Bruker spectrometer (Billerica), using the same solvent as internal standard. The multiplicities were determined by DEPT spectrum (Berger and Braun, 2004). COSY, HSQC, HMBC and NOESY spectra were recorded using Bruker microprograms. HR ESIMS spectra were recorded on a 6120 Quadrupole LC/MS instrument (Agilent Technologies). Analytical (0.25 mm thickness) and preparative TLC (0.50 mm thickness) were performed on silica gel (Kieselgel 60, F254) and on reversed phase (Kieselgel 60 RP-18, F254, 0.20 mm thickness) plates (Merck). Resulting spots were visualized by exposure to UV radiation (253 nm), or by spraying first with 10% H2SO4 in MeOH and then with 5% phosphomolybdic acid in EtOH, followed by heating at 110°C for 10 min. Column chromatography was performed using silica gel (Merck, Kieselgel 60, 0.063-0.200 mm).
**Fungus strain**

The *D. olivarum* strain used in this study was originally isolated from a cankered branch of lentisk collected in a natural area on Caprera Island (Italy). Representative genetic sequences from this strain were deposited in GenBank, with the accession numbers: ITS; KX833078), *tef1-α*; KX833079) and MAT1-2-1; MG015783 (Lopes et al., 2018). Pure cultures were maintained on potato dextrose agar (PDA) (Fluka, Sigma-Aldrich Chemie GmbH) and were stored at 4°C in the collection of the Dipartimento di Agraria, University of Sassari, Italy, as BL96.

**Production, extraction and purification of secondary metabolites**

*Diplodia olivarum* was grown on Czapek broth amended with 2% yeast extract or mineral salt medium (Pinkerton and Strobel, 1976), both at pH 5.7 in 1 L capacity Erlenmeyer flasks each containing 250 mL of medium. Each flask was seeded with 5 mL of a mycelium suspension and then incubated for 30 d at 25°C. Culture filtrates were obtained by filtering the cultures and then incubated for 30 d at 25°C. After filtration, the culture filtrate (10.0 L) obtained growing the fungus on modified mineral medium was extracted following the procedure described above to obtain 3.2 g of organic extract. This was fractioned by column chromatography on silica gel (80 × 4 cm) eluted with *n*-hexane-EtOAc (7.3), and yielded ten groups of homogeneous fractions. The residue of the third fraction (302.3 mg) was purified by column chromatography on silica gel (75 × 3 cm), eluted with *n*-hexane-CHCl₃-acetone (7.5:2:0.5), and yielded diplopiamarane (5: 1.4 mg, 0.01 mg L⁻¹) and (-)-mellein [6; 105.8 mg, 7.30 mg L⁻¹, Rf 0.65, eluent *n*-hexane-EtOAc-acetone (6:2.5:1.5)] as white solid, and a further amount of sphaeropsidin A (2; 33.2 mg, total yield 23.7 mg L⁻¹).

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**Spectroscopic data of secondary metabolites**

Oicleistanone (I): UV λ<sub>max</sub> (log ε) 333 (2.98), 241 (3.55) nm; IR ν<sub>max</sub> 1725, 1610, 1592, 1560, 1458 cm⁻¹; ¹H and ¹³C NMR: Table 1; HRESI-MS (+) spectrum m/z: 735 [2M+Na]⁺, 395 [M + K]⁺, 379.1876 [C<sub>22</sub>H<sub>28</sub>NaO₄, calcd. 379.1885, M+Na⁺], 311 [M+H₂CH₃CH₂OH]⁺. Sphaeropsidin A (2): [α]<sub>D</sub> = 18.3 (c 0.7, MeOH); [lit. (Evidente et al., 1997): [α]<sub>D</sub> = 16.8 (c 1.0, MeOH)]; ¹H NMR is very similar to that previously reported (Evidente et al., 1996); HRESI-MS (+) spectrum m/z: 715 [2M+Na]⁺, 369 [M+Na]⁺, 347.1820 [C<sub>28</sub>H<sub>27</sub>O₅, calcd. 347.1780, M+H⁺].

Sphaeropsidin C (3): [α]<sub>D</sub> = 18.3 (c 0.7, MeOH); [lit. (Evidente et al., 1997): [α]<sub>D</sub> = 16.8 (c 1.0, MeOH)]; ¹H NMR is very similar to that previously reported (Evidente et al., 1997); HRESI-MS (+) spectrum m/z:
703 [2M+K]^+, 687 [2M+Na]^+, 665 [2M+H]^+, 333.2037 [C_{20}H_{29}O_{4}, \text{calcd.} 333.2066, M+H]^+.

Sphaeropsidin G (4): [α]_{D}^{25} +48.6 (c 0.8, CHCl₃) [lit. (Cimmino et al., 2016): [α]_{D}^{25} +51.4 (c 0.56, CHCl₃)]; ¹H NMR is very similar to that previously reported (Cimmino et al., 2016); ESI-MS (+) spectrum m/z: 309 [M + K]^+, 293 [M + Na]^+, 271 [M + H]^+.

Diplopimarane (5): [α]_{D}^{25} +23.0 (c 0.1, CHCl₃) [lit. (Andolfi et al., 2014): [α]_{D}^{25} +25.8 (c 0.6, CHCl₃)]; ¹H NMR is very similar to that previously reported (Andolfi et al., 2014); ESI-MS (+) spectrum m/z: 623 [2M - 4H + Na]^+, 339 [M - 2H + K]^+, 325 [M + Na]^+, 323 [M - 2H + Na]^+.

(-)-Mellein (6): [α]_{D}^{25D} -93.0 (c 0.3 MeOH) [lit. (Masi et al., 2020): [α]_{D}^{25D} -90 (c 0.2, CH₃OH)]; ¹H NMR is similar to that previously reported (Masi et al., 2020); ESI MS (+) spectrum m/z: 179 [M + H]^+.

**Computational methods**

Molecular mechanics, Hartree-Fock (HF) and density functional theory (DFT) calculations were run with Spartan’18 (Wavefunction, Inc. 2018), with standard parameters and convergence criteria.

First, the conformers of (7S,15S)-1 and (7S,15R)-1 were investigated with the Monte Carlo algorithm using Merck molecular force field (MMFF). They were then screened by geometry optimizations at HF/3-21G level, single-point calculations at B3LYP/6-31G(d) level, and final geometry optimizations at the same level. Energies and populations were then estimated at the B97M-V/6-311+G(2df,2p) level. The procedure gave six energy minima for (7S,15S)-1 and ten minima for (7S,15R)-1 within the final energy threshold (10 kJ mol⁻¹ at the B97M-V/6-31G(d) level). ¹³C-NMR chemical shifts were then calculated with the GIAO method at the B3LYP/6-31G(d) level. An empirical correction was applied to each molecule depending on the number of bonds to the carbon and on the bond lengths (Hehre et al., 2019). ¹J coupling constants were determined as Boltzmann averages of all the DFT structures described above, either with Karplus equations or at B3LYP/pcJ-0 levels (Fermi contact term only).

**Leaf puncture assays**

Leaves of Phaseolus vulgaris L., Juglans regia L. and Quercus suber L. were used for this assay, and each compound was tested at 1.0 mg mL⁻¹. The assays were performed as previously reported (Andolfi et al., 2014), and each treatment was repeated three times. Leaves were observed daily and scored for symptoms after 5 d. The effects of the toxins on the leaves were observed up to 10 d. Lesions were estimated using APS Assess 2.0 software following the tutorials in the user’s manual. Lesion size was expressed in mm².

**Antifungal assays**

All compounds (1-6) were preliminarily tested on four different plant pathogens including the two fungi (Athelia rolfsii and D. corticola) and the two oomycetes (Phytophthora cambivora and P. lacustris). The sensitivity of all four species to these compounds was evaluated, depending on the species, on carrot agar (CA) or potato dextrose agar (PDA), as inhibition of the mycelium radial growth. The assays were performed as previously reported (Masi et al., 2016). Each metabolite was tested at 200 µg per plug. Methanol was used as negative controls. Metalaxyl-M (mefenoxam; p.a. 43.88%; Syngenta), a synthetic fungicide to which the oomycetes are sensitive, and PCNB (pentachloronitrobenzene) for ascomycetes and basidiomycetes, were used as positive controls. Each treatment consisted of three replicates, and the experiment was repeated two times.

**Artemia salina bioassays**

All compounds were assayed on brine shrimp larvae (Artemia salina L.). The assay was performed in cell culture plates with 24 cells (Corning) as previously described (Andolfi et al., 2014). The metabolites were tested at 100 mg mL⁻¹. Tests were performed in quadruplicate. The proportions (%) of larval mortality was determined after 36 h incubation at 27°C in the dark.

**RESULTS AND DISCUSSION**

The organic extract obtained from filtrates of D. olivavarum culture grown on Czapek medium was purified to yield a new nor-diterpenoid cleistanthane (1; Figure 2), named here as olicleistanone, together with two known pimarane diterpenoids identified as sphaeropsidins A and C (respectively 2 and 3; Figure 2), and two known nor-pimarane diterpenoids identified as sphaeropsidin G and diplopimarane (respectively, 4 and 5; Figure 2). When the fungus was grown on mineral salt medium it produced (-)-mellein (6; Figure 2), sphaeropsidin A (2) and low amounts of sphaeropsidin G (4) and diplopimarane (5)....
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The known compounds (2 to 6) were identified comparing their physical (specific optical rotation) and spectroscopic data (1H NMR and ESIMS) with those previously reported (Evidente et al., 1996; Evidente et al., 1997; Evidente et al., 2010; Andolfi et al., 2014; Abou-Mansour et al., 2015; Cimmino et al., 2016; Cimmino et al. 2017b; Masi et al., 2018; Masi et al., 2020).

Olicleistanone (1) has a molecular formula of \( C_{22}H_{28}O_4 \), as deduced from its HR ESIMS spectrum and consistent with nine hydrogen deficiencies. Preliminary investigation of its 1H and 13C NMR spectra (Table 1) showed that the compound is closely related to a tricyclic nor-diterpenoid, with aromatized and cyclohexadiene rings (C and B) joined to a dihydropyran ring (D) generated probably from a cleistanthane carbon skeleton (Devappa et al., 2011). The signal at \( \delta \) 195.5 in the 13C NMR spectrum also showed the presence of a conjugated ketone group (Breitmaier and Voelter, 1987). These results are in full agreement with the bands typical for carbonyl and aromatic groups observed in the IR spectrum (Najkanishi and Solomons 1977) and the absorption maxima observed in the UV spectrum (Pretsch et al., 2000).

The 1H and COSY spectra (Berger and Braun, 2004) of olicleistanone (1) showed the presence of the typical signals of two ortho-coupled protons (H-11 and H-12) of a 1,2,3,4-tetrasubstituted C benzene ring, and the singlets of a methoxy group (CH3-22), a vinyl methyl (CH3-17) and two methyls (CH3-19 and CH3-18) bonded to a quaternary carbon. The two methyls represent the head of the geranylgeranyl biosynthetic precursor which generated the diterpenoid cleistanthane carbon skeleton. The same spectra showed the signal of an ethoxy group. A signal pattern due to pyran moiety (ring D) of the benzohydrylpuran system (C and D rings) appeared as an ABC system. The spectra also showed a signal typical of the three adjacent methylene groups (CH2-1, CH2-2 and CH2-3) of the A ring (Pretsch et al., 2000).

The correlations observed in the HSQC spectrum (Berger and Braun, 2004) allowed the chemical shifts to be assigned to the protonated carbons, as reported in Table 1 (Breitmaier and Voelter, 1987).

The long range couplings observed in the HMBC spectrum (Berger and Braun, 2004) (Table 1) allowed the quaternary carbons to be assigned. The signals at \( \delta \) 34.0 correlated with H2-2, H2-3, H2-18 and H2-19 and were assigned to C-4, at 136.4 with H2-1, H2-3, H2-18 and H2-19 and assigned to C-5, at 195.5 with H2-1 and assigned to C-6, at 92.3 with H-15, H2-16 and H-20A and assigned to C-7, at 130.6 with H-12 and H2-17 and assigned to C-8, at 146.1 with H2-1, H2-2, H-11 and assigned to C-10, at 138.7 with H-11 and H2-17 and assigned to C-13, and at 130.2 with H-12, H-15 and H2-16 and assigned to C-14. The remaining signal at \( \delta \) 132.9 was assigned to C-9 (Breitmaier and Voelter, 1987). The correlation between C-7 and H-20A allowed the ethoxy group to be located at C-7 and consequently the methoxy group at C-15. Ethoxy groups are relatively rare in natural products, but not unprecedented, including several ethoxy-containing ketals like 1 (Wang et al., 2006; Lim et al., 2013; Xiong et al., 2015; Shen et al., 2015; Zhang et al., 2016). We avoided the use of ethanol during the extraction or purification process, which could lead to 1 as an artifact (Maltese et al., 2009; Capon, 2020).

Thus, the chemical shifts were assigned to all the carbons and the corresponding protons, which are reported in Table 1, and olicleistanone (1) was formulated as 4-ethoxy-6a-methoxy-3,8,8-trimethyl-4,5,8,9,10,11-hexahydrodibenzo[de,g]chromen-7(6aH)-one.
The structure assigned to 1 was supported by the other HMBC couplings reported in Table 1 and from the data of its HR ESIMS spectrum which showed the sodium dimer [2M+Na]+, the potassium [M+K]+ and the sodium [M+Na]+ adducts at m/z: 735, 395, 379.1876. The significant ion [M+H-CH3CH2OH]+ observed at m/z 311 was probably generated from a pseudo-molecular ion by loss of ethanol.

Attempts to assign the relative configuration of 1 were made recording a NOESY spectrum. The measured NOESY correlations are reported in Table 2, but since there is no clear correlation between the protons of the methoxy and ethoxy groups, these data alone were not sufficient to assign the relative configuration of the two chiral centres (C-7 and C-15). To better interpret NMR data, a molecular modelling study was undertaken. First, two diastereomeric structures (7S,15S)-1 and (7S,15R)-1 were generated and their possible conformations were explored by means of a conformational search with molecular mechanics (Merck molecular force field, MMFF). Geometry optimizations were then run with the density functional method (DFT) at the B97M-V/6-311+G(2df,2p)//B3LYP/6-31G(d) level, using the computational protocol for the prediction of 13C chemical shifts of flexible compounds, developed by Hehre et al. (2019). For the two diastereomers, six or ten conformers were found with detectable populations at room temperature. The various conformers differed in the conformation of the methoxy and ethoxy groups, but also in the puckering of ring A. A clear difference between the two diastereomers was the orientation of H-15, which was predominantly pseudo-equatorial in (7S,15S)-1 and pseudo-axial (7S,15R)-1. Thus, we presumed that the coupling constants between H-15 and H-16a/H-16b could be used to discriminate between the two isomers. Experimentally, H-15 appears as a doublet with splitting of 3.3 Hz, meaning that one J15/16 was small (3.3 Hz) and the other was negligible. This agreed with a pseudo-equatorial orientation. J15/16 were then estimated with Karplus curve and spin-spin coupling calculations at B3LYP/pcJ-0 level. These results are shown in Table 3, and strongly support the assignment of 1 as (7S*,15S*)-1. 13C-NMR calculations were then run at the B3LYP/6-31G(d) level. The estimated root-mean-square (rms) error between experimental and calculated 13C chemical shifts was acceptable (2.4–2.5)

| Table 1. 1H and 13C NMR and HMBC data for olicleistanone (1)a,b. |
|-----------------|-----------------|-----------------|-----------------|
| Position        | δC  (J in Hz)   | δH              | HMBC            |
| 1               | 27.4 t          | 2.77 (1H) dt    | H2-2, H2-3      |
| 2               | 18.6 t          | 1.80 (2H) m     | H2-1            |
| 3               | 40.5 t          | 1.62 (1H) m     | H2-2, H1-2, H2-18, H2-19 |
| 4               | 34.0 s          | 1.62 (1H) m     | H-15, H2-16, H-20A |
| 5               | 136.4 s         | 1.69 (1H) m     | H-12, H-17      |
| 6               | 195.5 s         | 1.69 (1H) m     | H-12, H-15, H-16 |
| 7               | 92.3 s          | 1.69 (1H) m     | H-15, H2-16, H-20A |
| 8               | 130.6 s         | 1.69 (1H) m     | H-12, H-17      |
| 9               | 132.9 s         | 1.69 (1H) m     | H-12, H-15, H-16 |
| 10              | 146.1 s         | 1.69 (1H) m     | H-15, H2-16, H-20A |
| 11              | 125.0 d         | 7.31 (1H) d     | H-12, H-15, H-16 |
| 12              | 131.3 d         | 7.31 (1H) d     | H-12, H-15, H-16 |
| 13              | 138.7 s         | 1.69 (1H) m     | H-11, H-17      |
| 14              | 130.2 s         | 1.69 (1H) m     | H-12, H-15, H-16 |
| 15              | 69.5 d          | 4.20 (1H) d     | H2-16, H2-22    |
| 16              | 60.5 t          | 4.22 (1H) d     | H-15            |
| 17              | 18.4 q          | 2.34 (3H) s     | H-12            |
| 18d             | 27.7 q          | 1.23 (3H) s     | H-19            |
| 19d             | 29.7 q          | 1.36 (3H) s     | H-18            |
| 20              | 59.0 t          | 3.33 (1H) dq    | H-21            |
| 21              | 15.4 q          | 3.33 (1H) dq    | H-21            |
| 22              | 55.7 q          | 3.33 (3H) s     | H-15            |

| Table 2. NOESY data for olicleistanone (1). |
|-----------------|-----------------|-----------------|-----------------|
| Irradiated      | Observed        | Irradiated      | Observed        |
| H-11            | H2-1            | OMe             | H2-17           |
| H2-20           | H2-17           | OMe             | H2-17           |
| H-15            | H2-17, OMe      |                 |                 |

| Table 3. Experimental and calculated JHH values (Hz) for olicleistanone (1)a. |
|-----------------|-----------------|-----------------|-----------------|
| Experimental    | Calculated      | Calculated      | Calculated      |
| J15/H-16a       | (Karplus)       | (DFT)           | (Karplus)       |
| 3.3             | 3.14            | 4.05            | 7.24            |
| H-15/H-16b      | 0.89            | 0.70            | 7.81            |
| n.d.            | 8.63            |                 |                 |

a 2D 1H, 1H (COSY) and 13C, 1H (HSQC) NMR experiments confirmed the correlations of all the protons and the corresponding carbons.
b Coupling constants (J) are given in parenthesis.
c Multiplicities were assigned with DEPT.
d These signals could be exchanged.
e These two signals are in part overlapped.

NMR data, a molecular modelling study was undertaken. First, two diastereomeric structures (7S,15S)-1 and (7S,15R)-1 were generated and their possible conformations were explored by means of a conformational search with molecular mechanics (Merck molecular force field, MMFF). Geometry optimizations were then run with the density functional method (DFT) at the B97M-V/6-311+G(2df,2p)//B3LYP/6-31G(d) level, using the computational protocol for the prediction of 13C chemical shifts of flexible compounds, developed by Hehre et al. (2019). For the two diastereomers, six or ten conformers were found with detectable populations at room temperature. The various conformers differed in the conformation of the methoxy and ethoxy groups, but also in the puckering of ring A. A clear difference between the two diastereomers was the orientation of H-15, which was predominantly pseudo-equatorial in (7S,15S)-1 and pseudo-axial (7S,15R)-1. Thus, we presumed that the coupling constants between H-15 and H-16a/H-16b could be used to discriminate between the two isomers. Experimentally, H-15 appears as a doublet with splitting of 3.3 Hz, meaning that one J15/16 was small (3.3 Hz) and the other was negligible. This agreed with a pseudo-equatorial orientation. J15/16 were then estimated with Karplus curve and spin-spin coupling calculations at B3LYP/pcJ-0 level. These results are shown in Table 3, and strongly support the assignment of 1 as (7S*,15S*)-1. 13C-NMR calculations were then run at the B3LYP/6-31G(d) level. The estimated root-mean-square (rms) error between experimental and calculated 13C chemical shifts was acceptable (2.4–2.5)
but similar for both isomers, confirming the $(7S^*,15S^*)$-1 assignment but without further supporting it. Nevertheless, we consider that the argument based on \(J\)-couplings is accurate enough to assign the relative configuration.

For the absolute configuration, the ECD spectrum of a solution of 1 in acetonitrile (1 mM, 0.01 cm cell) was measured. The ECD spectrum was not distinguishable from the baseline over the whole range (185-400 nm, data not shown), despite the optimal absorption (0.3 to 0.8 for the absorption peaks). It therefore must be concluded that the isolated sample of 1 was a racemate. Racemic natural products are rare, and are thought to result from nonenzymatic reactions (Zask and Ellestad, 2018). The chirality centre at C-7 of 1 is a tertiary benzyl carbon in a position to carbonyl group and it is therefore easily subject to racemization. However, racemization of this centre does not occur in a post-synthetic step, otherwise two diastereomers would be obtained. On the other hand, the isolated (7S,15S)-1 isomer was more stable than its (7S,15R) diastereomer by about 2 kcal mol\(^{-1}\) at the present level of calculation, suggesting that if the chiral centre at C-15 was biosynthesized in a later step than C-7, its configuration would be dictated by that at C-7.

All metabolites (1 to 6) isolated in this study were screened for phytotoxic, antifungal, antioomycete and zootoxic activities. Except for compound 2, phytotoxicity was not detected for any of the metabolites (at 1 mg mL\(^{-1}\)) when applied to leaves of Phaseolus vulgaris, Quercus suber, or Juglans regia. Sphaeropsidin A (2) caused necrotic lesions on leaves of all the plant species tested, with mean lesion sizes of 75.6 mm\(^2\) on P. vulgaris, 163.3 mm\(^2\) on J. regia and 15.1 mm\(^2\) on Q. suber.

In the assays of antifungal activity, sphaeropsidin A (2) inhibited mycelium growth of all the plant pathogens tested (100% inhibition rate). Diplopimarane (4) completely inhibited growth of Athelia rolfsii and partially inhibited growth of D. corticola, P. cambivora and P. lacustris, inhibition from 56% to 75%. No colony growth inhibition was observed for the other four metabolites at the concentration used.

In the brine shrimp larvae bioassay, which is widely used for toxicology and ecotoxicology studies, compounds 1, 4 and 6 (100 μg mL\(^{-1}\)) caused 100% larval mortality. Compound 2 caused 51% larval mortality, and compounds 3 and 5 were inactive.

Cleistanthane-type diterpenoids are produced by different fungi and plants, but few examples of cleistanthane nor-diterpenoids are reported. Among them there are aspergiloids A, B, F and G isolated from the fermentation broth extract of Aspergillus sp. YXF3, an endophytic fungus from Ginkgo biloba. However, no biolog-
M. A. Cimmino et al., 2015; Barilli et al., 2017; Aznar et al., 2019). Drug-based phytoalexins can also be used in medicine against some important human diseases, such as cancer, malaria, dengue and yellow fever, and against fungal and bacterial infections (Bajsa et al., 2007; Evidente et al., 2014, Masi et al. 2017; Masi et al., 2018; Roscetto et al., 2020). Some of these toxins could also be produced in industrial large scale, and be formulated for applications in agriculture and medicine. Among the toxins previously isolated, and also from D. olivarum, sphaeropsidin A (2) is a phytoxin with strong potential for drug development (Ingles et al., 2017; Masi et al., 2018; Roscetto et al., 2020).

In conclusion, this study was the first to investigate secondary metabolites produced by D. olivarum, an emerging pathogen of forest trees in the Mediterranean region. The results confirm that Botryosphaeriaceae are sources of bioactive secondary metabolites, some of which have potential for applications in biotechnology sectors.

Among the metabolites produced in vitro by D. olivarum, sphaeropsidin A and diplopimarane inhibited vegetative growth of four plant pathogens belonging to different phyla. Additionally, the strong activity of the newly identified metabolite, olicleistanone (1), against A. salina deserves detailed investigation, because several applications of A. salina in toxicology and ecotoxicology continue to be widely used.

ACKNOWLEDGEMENTS

This study was partially supported by the “Fondo di Ateneo per la ricerca 2019”, internal funding provided by the University of Sassari. Antonio Evidente is associated with the Istituto di Chimica Biomolecolare del CNR, Pozzuoli, Italy.

SUPPORTING INFORMATION

1D and 2D NMR data for 1 and HRESI-MS spectra of 1-3.

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