

Induction of Haustorium Development by Sphaeropsidones in Radicles of the Parasitic Weeds Striga and Orobanche. A Structure-Activity Relationship Study

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# 1 Induction of Haustorium Development by Sphaeropsidones in 2 Radicles of the Parasitic Weeds *Striga* and *Orobanche*. A Structure– 3 Activity Relationship Study

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12 **ABSTRACT:** Crop attack by parasitic weeds such as *Striga* and *Orobanche* occurs through developmental processes triggered by  
 13 host chemodetection. Seeds of those weed species remain dormant in the soil until germination is triggered by host root  
 14 exudates. The development of haustorium, a parasitic plant organ that invades the host to withdraw its nutrients, is also initiated  
 15 in Orobanchaceae by host molecular cues. The induction of haustorium development by exogenous signals has previously been  
 16 reported for *Striga* but not for *Orobanche* species. In this work, we demonstrate that sphaeropsidone and *epi*-sphaeropsidone, two  
 17 phytotoxic cyclohexene oxides isolated from the fungus *Diplodia cupressi*, a causal agent of cypress canker, induce haustorium  
 18 development in radicles of the parasitic weeds *Striga hermonthica*, *Orobanche crenata*, and *Orobanche cumana*. This is the first  
 19 report of chemical stimulation of haustorium development in radicles of *Orobanche* in the absence of host. In addition, SAR  
 20 studies were carried out by testing the haustorium-inducing activity of the natural cyclohexene oxides, seven already known and  
 21 four new hemisynthetic derivatives, in *O. cumana*, *O. crenata*, and *S. hermonthica*, to find a molecular specificity model required  
 22 for haustorium induction. The results suggested that the haustorium-inducing activity is due to the possibility to convert the  
 23 natural sphaeropsidone and natural and hemisynthetic derivatives in the corresponding 4-methoxyquinone and that the  
 24 stereochemistry at C-5 also seems to affect this activity.

25 **KEYWORDS:** *Striga hermonthica*, *Orobanche crenata*, *Orobanche cumana*, cyclohexene oxides, attachment organ,  
 26 haustorium-inducing factor

## 27 ■ INTRODUCTION

28 Root parasitic plants have evolved to exploit another plant root  
 29 system to fulfill their water and nutrient requirements. Two  
 30 main types of parasitic plants can be recognized regarding their  
 31 photosynthetic status: hemiparasitic plants perform some  
 32 degree of photosynthesis but are dependent on the host mainly  
 33 for water and inorganic nutrients, while holoparasitic plants lack  
 34 of photosynthetic machinery and in the nutrient diversion from  
 35 the host they include all their required reduced carbon.  
 36 Hemiparasitic plants are also classified in obligated or  
 37 facultative parasites depending on their ability to develop an  
 38 autotrophic way of life in the absence of a suitable host.<sup>1</sup> Plants  
 39 from up to 28 dicotyledonous families use the plant-parasitic  
 40 strategy to obtain competitive advantage from neighboring host  
 41 plants.<sup>2</sup> In all of them, the core feature of their parasitic strategy  
 42 is a specialized host-invasive organ called haustorium that  
 43 develops sequential functions of host attachment, penetration,  
 44 and connection with host vascular tissues.<sup>3,4</sup> At least in one  
 45 family of parasitic plants, the Orobanchaceae, the haustorium  
 46 development is initiated by host-derived metabolites.<sup>5</sup> Some  
 47 members of this family, i.e., the hemiparasitic plants *Striga* spp.  
 48 and the holoparasitic plants *Orobanche* spp., are weeds to which  
 49 despite their high negative economic impact in agriculture there

is not effective and or practicable control and thus the  
 development of innovative control strategies is urgent.

In vitro screenings of host-derived factors that initiate  
 developmental processes in parasitic plants are essential in  
 breeding programs for parasitic plant resistance.<sup>6</sup> *Striga*  
 haustorium can be monitored in vitro by exposing *Striga*  
 seedlings to inducing factors. Upon detection of haustorium-  
 inducing factors, the *Striga* seedling initiates haustorium  
 development and undergoes quick morphological changes  
 that prepare it for its first step in the host invasion process:  
 attachment to host root. Long haustorial hairs with functions of  
 adhesion and attachment develop from elongation of epidermal  
 cells surrounding the haustorium apex.<sup>4</sup> Despite the fact that  
*Striga* and *Orobanche* haustoria have a common evolutionary  
 origin,<sup>7</sup> fundamental differences exist at their host-preattached  
 haustorial stage. Morphologically, *Orobanche* haustorium is by  
 far less defined. It lacks haustorial hairs but rather it develops

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67 short secretory papillae with function of the adhesion surface.<sup>8</sup>  
 68 Another fundamental difference with *Striga* is that in *Orobanche*  
 69 species, the chemistry of haustorium initiation is completely  
 70 unknown. Several authors have suggested that *Orobanche*  
 71 species do not require host chemical factors for haustorium  
 72 development, which would make *Orobanche* the notable  
 73 exception in the Orobanchaceae.<sup>8–10</sup> In consequence, in vitro  
 74 assays of haustorium-inducing factors or haustorium-inducing  
 75 root exudates have been unfeasible in *Orobanche*, hampering  
 76 the screening for low-inducer genotypes in crop germplasm  
 77 collections.

78 Several phenols, flavonoids, and quinones have been  
 79 identified as factors inducing both terminal and lateral haustoria  
 80 in obligated hemiparasitic plants, i.e., *Striga hermonthica* and  
 81 facultative hemiparasitic plants, i.e., *Triphysaria versicolor*.<sup>4,13–15</sup>  
 82 The Atsatt's hypothesis<sup>16</sup> that parasitic plants evolved to use  
 83 defense metabolites as host recognition signals is supported by  
 84 the fact that many haustorium-inducing factors are structurally  
 85 similar to phytotoxins produced by allelopathic plants. There is  
 86 a similar gene expression in parasitic roots when they are  
 87 exposed to host contact or allelochemicals.<sup>5,16</sup> The haustorium  
 88 development in hemiparasitic Orobanchaceae is under redox  
 89 control mediated by cycling between the reduced and oxidized  
 90 states of the haustorial-inducing compounds.<sup>17</sup> Similar models  
 91 in the quinone-mediated actions of both phytotoxic allelopathy  
 92 and haustorium initiation are proposed.<sup>5,10,18</sup>

93 Some fungal metabolites are able to interfere with weed  
 94 parasitism either by phytotoxic action on germination or  
 95 growth inhibition or by triggering developmental programs in a  
 96 suicidal fashion, i.e., in the absence of a host. These new  
 97 compounds constitute alternative candidate ingredients for the  
 98 development of new herbicides. Parasitic weeds that can be  
 99 targeted include species of high economic important members  
 100 of *Orobanche*, *Phelipanche*, and *Striga* genera.<sup>11,12,19–22</sup>

101 Sphaeropsidone and *epi*-sphaeropsidone (**1** and **2**, Figure 1)  
 102 are two cyclohexene oxides isolated together, several

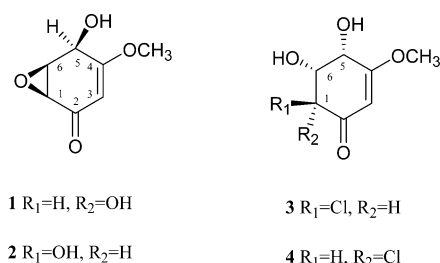


Figure 1. Structures of sphaeropsidone and *epi*-sphaeropsidone (**1** and **2**) and chlorosphaeropsidone and epichlorosphaeropsidone (**3** and **4**).

103 sphaeropsidins<sup>23</sup> as the main phytotoxins produced from  
 104 *Diplodia cupressi*, the causal agent of cypress (*Cupressus*  
 105 *sempervirens* L.) canker in the Mediterranean basin.<sup>24</sup>  
 106 Successively, two chlorinated cyclohexenones, closely related  
 107 to **1** and **2** and named chlorosphaeropsidone and epichlorop-  
 108 sphaeropsidone (**3** and **4**, Figure 1), were isolated from the  
 109 same fungal culture filtrates.<sup>25</sup> They have phytotoxic activity  
 110 causing necrosis and wilting in *Quercus* species and in tomato  
 111 and antifungal activity by inhibiting mycelial growth in species  
 112 of *Phytophthora*.<sup>26</sup>

113 In a previous study, among fungal and plant phytotoxins,  
 114 sphaeropsidones were identified as metabolites able to induce  
 115 morphological changes in the *Orobanche* radicle that resemble

the attachment organ.<sup>11</sup> Because of the nuanced morphology of  
 116 *Orobanche* preattached haustorium and to the fact that no  
 117 chemicals with haustorium-inducing activity have been  
 118 previously identified for any *Orobanche* species, we have  
 119 included in the present work *S. hermonthica* as a living marker  
 120 for haustorium induction due to its very distinct haustorium  
 121 morphology. The present article confirms the haustorium-  
 122 inducing nature of sphaeropsidone activity and that it acts  
 123 across the parasitic weed genera *Orobanche* and *Striga*.  
 124 Haustorium-inducing assays of sphaeropsidones were per-  
 125 formed in *O. cumana*, *O. crenata*, and *S. hermonthica*. In  
 126 addition, structure–activity relationships were carried out by  
 127 assaying two natural analogues (**3** and **4**), seven already known  
 128 and four new sphaeropsidones derivatives<sup>26</sup> prepared by  
 129 chemical transformation of **1** and **2**.  
 130

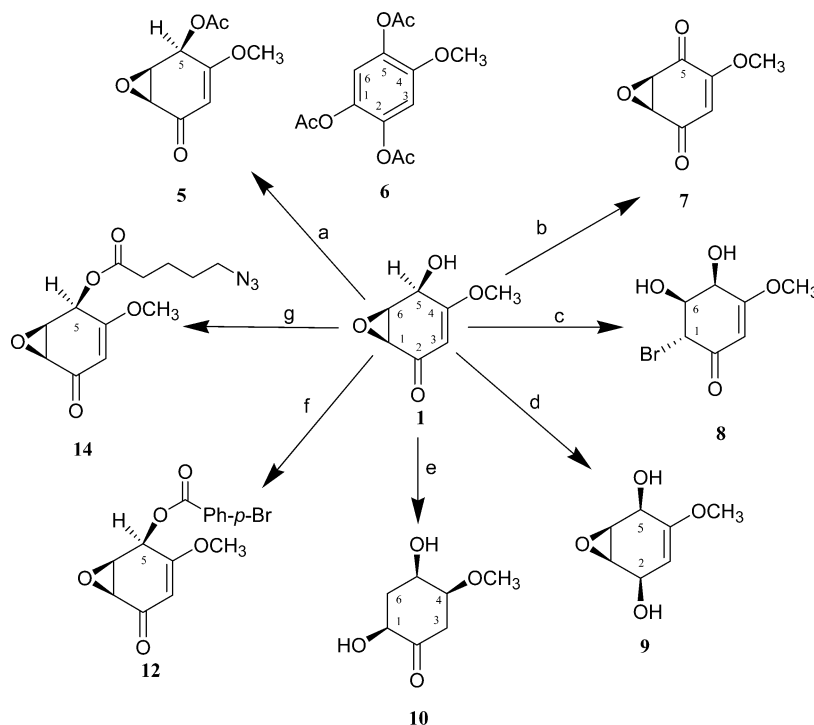
## MATERIALS AND METHODS

131  
 132 **General Experimental Procedures.** IR spectra were recorded as  
 133 deposit glass film on a 5700 FT-IR spectrometer (Thermo Electron  
 134 Corp. Nicolet, Madison, WI, USA), and UV spectra were measured in  
 135 MeCN on a V-530 spectrophotometer (Jasco, Tokyo, Japan). <sup>1</sup>H and  
 136 <sup>13</sup>C NMR spectra were recorded at 400/100 or 500/125 MHz in  
 137 CDCl<sub>3</sub> on Bruker spectrometers (Karlsruhe, Germany). The same  
 138 solvent was used as internal standard. Carbon multiplicities were  
 139 determined by DEPT spectra.<sup>27</sup> ESI spectra were recorded on a 6230  
 140 TOF LC/MS instrument (Agilent Technologies, Milan, Italy).  
 141 Analytical and preparative TLC were performed on silica gel (Kieselgel  
 142 60, F<sub>254</sub>, 0.25 and 0.5 mm, respectively) plates (Merck, Darmstadt,  
 143 Germany). The spots were visualized by exposure to UV radiation  
 144 (253) or by spraying first with 10% H<sub>2</sub>SO<sub>4</sub> in MeOH and then with  
 145 5% phosphomolybdic acid in EtOH, followed by heating at 110 °C for  
 146 10 min. Column chromatography was performed using silica gel  
 147 (Kieselgel 60, 0.063–0.200 mm) (Merck).

148 **Fungal Strains.** The *D. cupressi* strain used in this study was  
 149 purchased from Centraalbureau voor Schimmelcultures of Baarn  
 150 (Netherlands). Pure culture was maintained on potato dextrose agar  
 151 (PDA) (Fluka, Sigma-Aldrich Chemic GmbH, Buchs, Switzerland)  
 152 and stored at 4 °C in the collection of Dipartimento di Agraria,  
 153 Università di Sassari, Italy, as 261.85 CBS. The fungus was grown as  
 154 previously reported.<sup>26</sup>

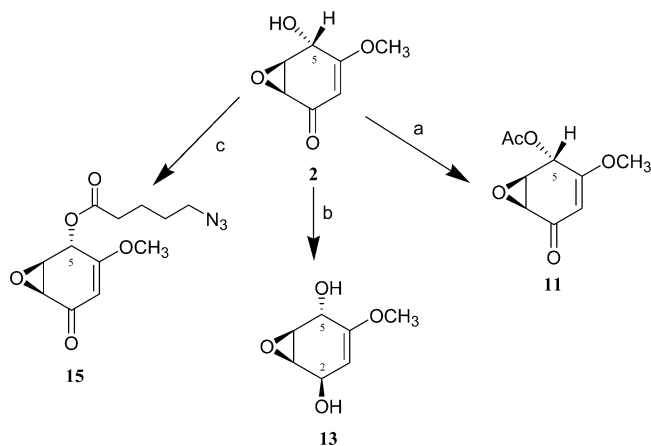
155 **Extraction and Isolation of Sphaeropsidones.** One-week-old  
 156 colonies of the fungus on PDA in 9 cm Petri dish were cut off and  
 157 blended for 30 s with 100 mL of sterile distilled water to prepare the  
 158 inoculum for liquid fermentation. Each 2 L Erlenmeyer flask  
 159 containing 400 mL of modified Czapek medium supplemented with  
 160 2% corn meal (pH 5.7) was seeded with 5 mL of the mycelial  
 161 suspension and then incubated at 25 °C for 4 weeks in darkness. The  
 162 culture filtrates (15 L) were acidified and extracted exhaustively with  
 163 EtOAc as previously reported.<sup>24</sup> The organic extract, obtained as a  
 164 brown–red oil (9.2 g), was chromatographed on a silica gel column  
 165 (110 cm × 5 cm) eluted with 3 L of CHCl<sub>3</sub>/*i*-PrOH (9:1, v/v),  
 166 affording nine groups of homogeneous fractions. The residues (3.6 g)  
 167 of fractions 4–7 were combined and further purified by a silica gel  
 168 column (90 cm × 4 cm) eluted with 2.5 L of CHCl<sub>3</sub>/*i*-PrOH (9:1, v/  
 169 v), yielding six groups of homogeneous fractions. The residue of  
 170 fraction 3 was crystallized from EtOAc/*n*-hexane (1:5, v/v), yielding  
 171 sphaeropsidone, **1** (*R*<sub>f</sub> 0.40, 2.3 g, 153.3 mg/L) (Figure 1), as white  
 172 needles. The mother liquors were further purified by column  
 173 chromatography on silica gel (75 cm × 3 cm) eluted with 1 L of  
 174 petroleum ether–Me<sub>2</sub>CO (7:3, v/v), affording *epi*-sphaeropsidone, **2**  
 175 (*R*<sub>f</sub> 0.53, 725 mg, 48.3 mg/L) (Figure 1), as a homogeneous oil.  
 176 Chlorosphaeropsidone, **3**, and epichlorosphaeropsidone, **4** (Figure 1),  
 177 were purified from the same culture filtrate, and the purification  
 178 procedures for these compounds have been described previously.<sup>25</sup>

179 **Preparation of Sphaeropsidones Derivatives (5–11).** The 5-  
 180 *O*-acetyl, **5**, 2,4,5-triacetylanisole, **6**, 1,4-dione, **7**, bromohydrin, **8**, 1,4-  
 181 diol, **9**, and 2,4-dihydroxycyclohexanone, **10**, derivatives of sphaerop-  
 182 sidone, **1** (Scheme 1) as well as the 5-*O*-acetyl derivative, **11**, of *epi*-  
 182

Scheme 1. Synthesis of Sphaeropsidone Derivatives (5–10, 12, and 14)<sup>a</sup>

<sup>a</sup>Reagents and conditions: (a) Ac<sub>2</sub>O, pyridine, 80 °C; (b) MnO<sub>2</sub>, CH<sub>2</sub>Cl<sub>2</sub>, rt; (c) Li<sub>2</sub>NiBr<sub>4</sub>, THF, rt; (d) NaBH<sub>4</sub>, MeOH, rt; (e) H<sub>2</sub>, Pd 10%, MeOH, rt; (f) CH<sub>3</sub>CN, DMAP, BrC<sub>6</sub>H<sub>4</sub>COCl, rt; (g) CH<sub>2</sub>Cl<sub>2</sub>, pyridine, DCC, C<sub>5</sub>H<sub>9</sub>N<sub>3</sub>O<sub>2</sub>, 0 °C 1 h and rt 2 h.

183 sphaeropsidone, **2** (Scheme 2), were prepared according to the  
184 procedures previously reported.<sup>26</sup>

Scheme 2. Synthesis of *epi*-Sphaeropsidone Derivatives (11, 13, and 15)<sup>a</sup>

<sup>a</sup>Reagents and conditions: (a) Ac<sub>2</sub>O, AcONa, 80 °C; (b) NaBH<sub>4</sub>, MeOH, rt; (c) CH<sub>2</sub>Cl<sub>2</sub>, pyridine, DCC, C<sub>5</sub>H<sub>9</sub>N<sub>3</sub>O<sub>2</sub>, 0 °C 1 h and rt 2 h.

185 **5-O-*p*-Bromobenzoyl Ester of Sphaeropsidone (12).** Sphaer-  
186 opsidone, **1** (5 mg), was dissolved in CH<sub>3</sub>CN (0.5 mL), and DMAP  
187 (4-dimethylaminopyridine) (10 mg) and *p*-bromobenzoyl chloride (10  
188 mg) were added. The reaction mixture was stirred at room  
189 temperature for 4 h and then evaporated under reduced pressure.  
190 The residue (10.0 mg) was purified by TLC on silica gel eluted with  
191 CHCl<sub>3</sub>/*i*-PrOH (95:5, v/v), giving derivatives **12** (3.65 mg, R<sub>f</sub> 0.78)  
192 (Scheme 1, as uncolored oil. **12** had: UV λ<sub>max</sub> (log ε) 250 (4.07) nm.  
193 IR ν<sub>max</sub> 1728, 1670, 1618, 1590 cm<sup>-1</sup>. <sup>1</sup>H NMR, δ: 8.01 (d, J = 8.7 Hz,

H-2' and H-6'), 7.63 (d, J = 8.7 Hz, H-3' and H-5'), 6.19 (d, J = 2.5  
194 Hz, H-5), 5.35 (br s, H-3), 3.87 (dd, J = 4.3 and 2.5, H-6), 3.71 (s,  
195 OMe), 3.54 (dd, J = 4.3 and 1.7, H-1). ESIMS (+) *m/z* 364 [M + 2 +  
196 Na]<sup>+</sup>, 362 [M + Na]<sup>+</sup>, 342 [M + 2 + H]<sup>+</sup>, 340 [M + H]<sup>+</sup>.

**5-O-5'-Azidopentanoyl Ester of Sphaeropsidone (14).** To  
198 compound **1** (5.0 mg) dissolved in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (4.5 mL) and  
199 pyridine (100 mL) were added DCC (*N,N'*-dicyclohexylcarbodiimide)  
200 (5 mg) and 5-azidopentanoic acid (20 μL). The reaction was left at 0  
201 °C for 1 h and then at room temperature for 2 h. The reaction was  
202 stopped by evaporation under N<sub>2</sub>. The residue (10 mg) was purified  
203 by TLC on silica gel eluted with CHCl<sub>3</sub>/*i*-PrOH (95:5, v/v), giving  
204 derivatives **14** (7.90 mg, R<sub>f</sub> 0.74) (Scheme 1), as uncolored oil. **14** had:  
205 UV λ<sub>max</sub> (log ε) 253 (3.51) nm. IR ν<sub>max</sub> 2100, 1746, 1672, 1618 cm<sup>-1</sup>.  
206 <sup>1</sup>H NMR, 5.97 (d, J = 2.9 Hz, H-5), 5.30 (br s, H-3), 3.76 (dd, J = 4.0  
207 and 2.9, H-6), 3.70 (s, OMe), 3.50 (dd, J = 4.0 and 1.4, H-1), 3.32 (t, J  
208 = 6.5 Hz, CH<sub>2</sub>-5'), 2.53 (t, J = 7.3 Hz, CH<sub>2</sub>-2'), 1.83–1.76 (m, CH<sub>2</sub>-  
209 3'), 1.71–1.64 (m, CH<sub>2</sub>-4'). ESIMS (+) *m/z* 304 [M + Na]<sup>+</sup>, 282 [M  
210 + H]<sup>+</sup>.

**NaBH<sub>4</sub> Reduction of *epi*-Sphaeropsidone (13).** To *epi*-  
212 Sphaeropsidone, **2** (5 mg), dissolved in MeOH (15 mL), was added  
213 NaBH<sub>4</sub> (5 mg) under stirring at room temperature for 30 min. The  
214 mixture was neutralized with 0.1 M HCl, extracted with CH<sub>2</sub>Cl<sub>2</sub> (3 ×  
215 30 mL) and dried (Na<sub>2</sub>SO<sub>4</sub>). The oily residue was purified by  
216 preparative TLC, using CHCl<sub>3</sub>-*i*-PrOH (9:1) for elution to give the  
217 derivative **13** as white needles (3.45 mg, R<sub>f</sub> 0.37) (Scheme 2). **13** had:  
218 UV λ<sub>max</sub> (log ε) < 220 nm. IR ν<sub>max</sub> 3413, 1671 cm<sup>-1</sup>. <sup>1</sup>H NMR, δ: 4.64  
219 (br d, J = 10.4 Hz, H-2), 4.57 (br s, H-3), 4.39 (br s, H-5), 3.57 (s,  
220 OMe), 3.53 (br s, H-6), 3.50 (br s, H-1), 2.25 (d, J = 4.4 Hz, HO-  
221 C(5)), 1.84 (d, J = 10.4 Hz, HO-C(2)). ESIMS (+) *m/z* 181 [M +  
222 Na]<sup>+</sup>, 159 [M + H]<sup>+</sup>.

**5-O-5'-Azidopentanoyl Ester of *epi*-Sphaeropsidone (15).** To  
224 compound **2** (5.0 mg) dissolved in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (4.5 mL) and  
225 pyridine (100 mL) were added DCC (5.0 mg) and 5-azidopentanoic  
226 acid (20 μL). The reaction was left at 0 °C for 1 h and then at room  
227 temperature for 2 h. The reaction was stopped by evaporation under  
228 N<sub>2</sub>. The residue (10.0 mg) was purified by TLC on silica gel eluted  
229 with CHCl<sub>3</sub>/*i*-PrOH (95:5, v/v) giving derivatives **15** (7.90 mg, R<sub>f</sub> 230

231 0.74) (Scheme 2) as uncolored oil. **15** had: UV  $\lambda_{\text{max}}$  (log  $\epsilon$ ) 249 (3.48)  
 232 nm. IR  $\nu_{\text{max}}$  2098, 1749, 1671, 1624  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR, 5.92 (br s, H-5),  
 233 5.36 (br s, H-3), 3.70 (s, OMe), 3.59 (br s, H-6), 3.45 (br s, H-1), 3.31  
 234 (t,  $J = 6.5$  Hz,  $\text{CH}_2\text{-5}'$ ), 2.45 (t,  $J = 7.2$  Hz,  $\text{CH}_2\text{-2}'$ ), 1.80–1.72 (m,  
 235  $\text{CH}_2\text{-3}'$ ), 1.68–1.71 (m,  $\text{CH}_2\text{-4}'$ ). ESIMS (+)  $m/z$  304 [ $\text{M} + \text{Na}$ ] $^+$ ,  
 236 282 [ $\text{M} + \text{H}$ ] $^+$ .

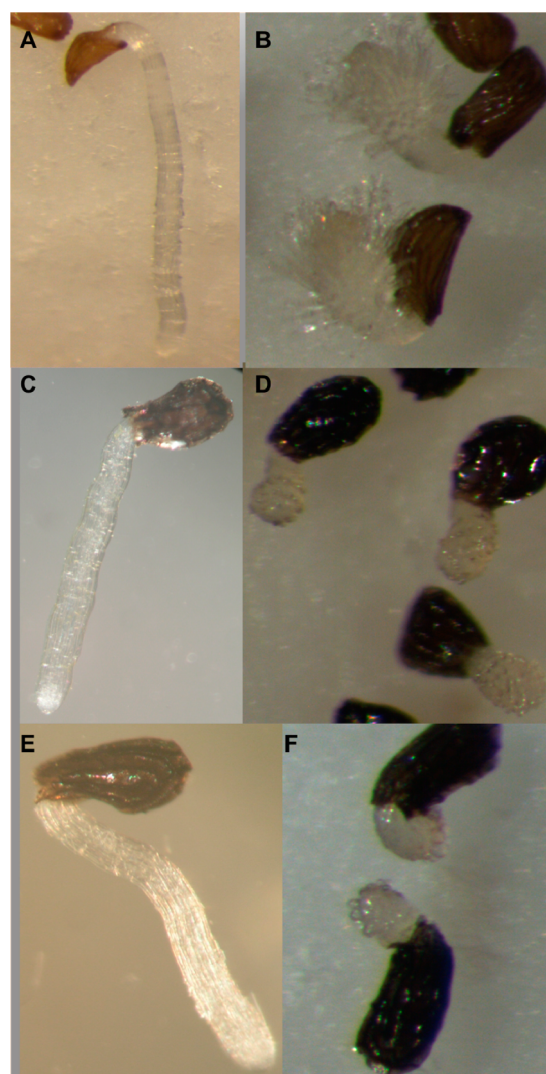
237 **Seed Conditioning.** The haustorium-inducing activity of sphaer-  
 238 opsiones was tested on parasitic seedlings, and the starting material  
 239 for the haustorium bioassays were parasitic seeds. *Striga* and *Orobanch*  
 240 seeds require chemical stimulation to germinate but prior they become  
 241 responsible to germination-inducing factors these seeds require a  
 242 period of warm stratification called conditioning.<sup>28</sup> To achieve that, *O.*  
 243 *crenata*, *O. cumana*, and *S. hermonthica* seeds were surface sterilized by  
 244 immersion in 0.5% (w/v) NaOCl and 0.02% (v/v) Tween 20, were  
 245 sonicated for 2 min, rinsed thoroughly with sterile distilled water, and  
 246 dried in a laminar air flow cabinet. To allow seed conditioning,  
 247 approximately 100 seeds of each species were placed separately in 9  
 248 mm diameter glass fiber filter paper disks (GFFP) moistened with 50  
 249  $\mu\text{L}$  of sterile distilled water and placed inside Petri dishes in incubators  
 250 during 14 days at 30 °C for *S. hermonthica* and 10 days at 22 °C for *O.*  
 251 *crenata* and *O. cumana*.

252 **Haustrorium-Inducing Assay.** A solution of the germination  
 253 stimulant GR24<sup>29</sup> was prepared at  $10^{-6}$  M in sterile distilled water.  
 254 Immediately before use, stock solutions in methanol of the candidate  
 255 haustorial-inducing compounds as sphaeropsidone, **1**, *epi*-sphaerop-  
 256 sidone, **2**, their natural analogues, **3** and **4**, and hemisynthetic  
 257 derivatives, **5–15**, were diluted in the GR24 solution at five decreasing  
 258 concentrations of sphaeropsidone (100, 10, 1, 0.1, 0.01  $\mu\text{M}$ ) on *Striga*  
 259 *hermonthica* and three decreasing concentrations of sphaeropsidone  
 260 (100, 10, 1  $\mu\text{M}$ ) on *Orobanch* spp. but keeping constant the  
 261 concentration of GR24 and methanol in order to allow comparisons.  
 262 Final concentration of methanol was adjusted to 0.70% (v/v). In these  
 263 conditions, the solutions of natural sphaeropsidones and derivatives  
 264 had a pH value of 6.5. GFFP disks containing the conditioned *Striga*  
 265 and *Orobanch* seeds were transferred inside a laminar flow cabinet to  
 266 sterile filter paper in order to remove the excess of water used for seed  
 267 conditioning and then transferred to new 10 cm sterile Petri dishes.  
 268 Then, each disk was treated with a 50  $\mu\text{L}$  aliquot of the respective test  
 269 solution. GR24 solution (containing 0.70% methanol) was used as  
 270 negative control. Seeds were incubated in the dark at 30 °C (*S.*  
 271 *hermonthica* seeds) or at 22 °C (*O. crenata* and *O. cumana* seeds).  
 272 Because of the faster germination of *Striga* when compared with  
 273 *Orobanch* species, haustorium formation was examined at 3 days in *S.*  
 274 *hermonthica* and 6 days in *O. crenata* and *O. cumana*. Using a  
 275 stereoscopic microscope at 30 $\times$  magnification, the percentage of  
 276 haustorium formation in emerged radicles of each species was  
 277 established.

278 **Statistical Analysis.** The bioassays were performed twice with  
 279 three replicates. Percentage data were approximated to normal  
 280 frequency distribution by means of angular transformation ( $180/\pi \times$   
 281  $\arcsin(\sqrt{[\%/100]})$ ) and subjected to analysis of variance (ANOVA)  
 282 using SPSS software for Windows, version 21.0 (SPSS Inc., Chicago,  
 283 Illinois, USA). The significant of mean differences between each  
 284 treatment against the negative control was evaluated by the two-sided  
 285 Dunnett test. Null hypothesis was rejected at the level of 0.05.

## 286 ■ RESULTS AND DISCUSSION

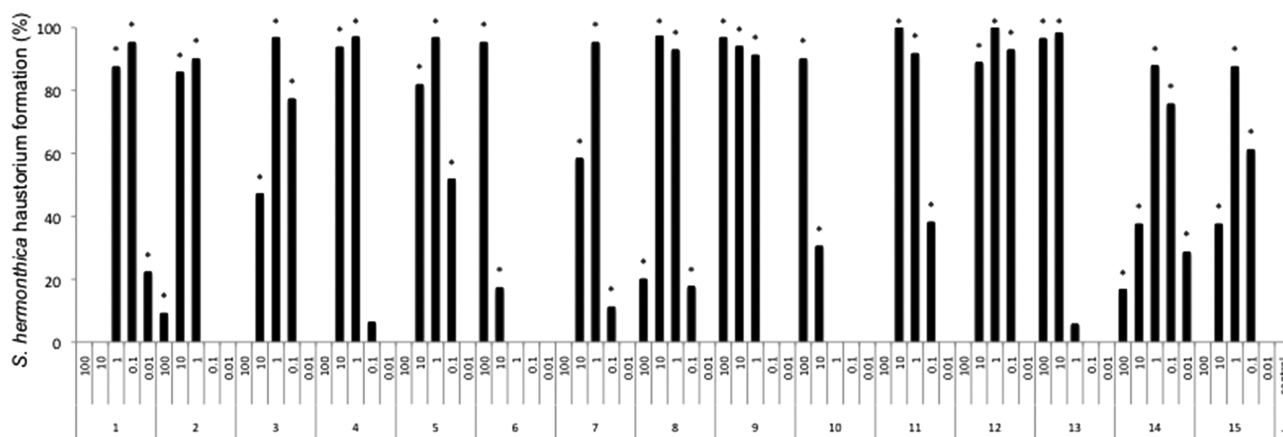
287 Radicles of *O. crenata* and *O. cumana* did not develop  
 288 haustorium when treated with the negative control test solution  
 289 GR24 (Figure 2C,E). This finding is in agreement with  
 290 observations of radicles made by Cimmino et al. (2014)<sup>21</sup> but  
 291 in contradiction with the generalized view suggesting that  
 292 radicle of *Orobanch* seedlings spontaneously initiate typical  
 293 attachment organs in the absence of exogenous signals.<sup>8–10</sup> *S.*  
 294 *hermonthica* radicles exposed to negative control also lacked  
 295 haustorial development (Figure 2A). The spontaneous develop-  
 296 ment of terminal haustorium does not make biological sense for  
 297 obligated parasitic weeds such as *Orobanch* and *Striga* as it will



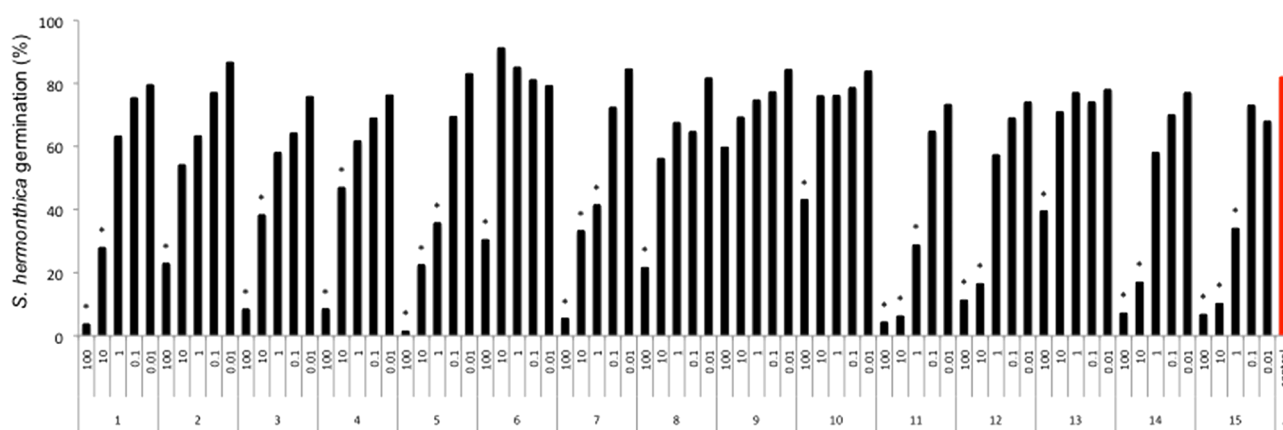
**Figure 2.** Haustorium formation in (A) *Striga hermonthica* seedlings exposed to negative control, (B) *S. hermonthica* seedlings exposed to sphaeropsidone, (C) *Orobanch crenata* seedlings exposed to negative control, (D) *O. crenata* seedlings exposed to sphaeropsidone, (E) *Orobanch cumana* seedlings exposed to negative control, (F) *O. cumana* seedlings exposed to sphaeropsidone.

threaten the success of host attack. Because the root meristem  
 does not resume its normal growth after terminal haustorium  
 development, a haustorium initiated before host contact  
 impedes successful host attack, leading to the death of the  
 parasitic seedling. In this work instead of spontaneous  
 haustorium development, specificity for the chemistry that  
 initiates terminal haustorium development was expected and  
 was confirmed for *Orobanch* as well as is the case for *Striga*.

Sphaeropsidone and *epi*-sphaeropsidone, phytotoxins pro-  
 duced by *D. cupressi*,<sup>26</sup> were strong inducers of haustoria in *S.*  
*hermonthica* radicles. Recognition of sphaeropsidones by  
 radicles of *S. hermonthica* promotes a cessation in the growth  
 of the parasitic radicle and the development of attachment  
 organ at the radicle tips that is in agreement with the  
 description of terminal haustorium made by Riopel and Timko  
 (1995).<sup>4</sup> More than 80% of *S. hermonthica* radicles developed  
 haustorium when exposed to sphaeropsidone at concentrations  
 between 1 and 0.1  $\mu\text{M}$  or to *epi*-sphaeropsidone at 315  
 concentration between 10 and 1  $\mu\text{M}$  (Figures 2B and 3). For 316



**Figure 3.** Induction of *S. hermonthica* haustorium formation by sphaeropsidone, 1, *epi*-sphaeropsidone, 2, two natural analogues, 3 and 4, eight sphaeropsidones derivatives, 5–10, 12, and 14, and three *epi*-sphaeropsidone derivatives, 11, 13, and 15, tested in the range 0.01–100  $\mu\text{M}$ . \*Indicates differences at the 0.05 level compared with the control.



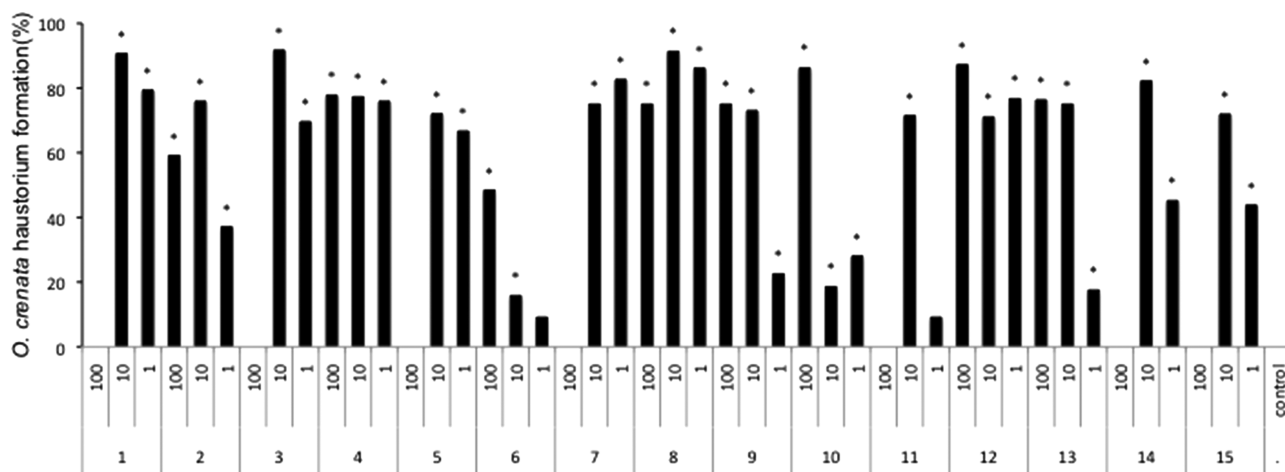
**Figure 4.** Inhibition of *S. hermonthica* germination by sphaeropsidone, 1, *epi*-sphaeropsidone, 2, two natural analogues, 3 and 4, eight sphaeropsidones derivatives, 5–10, 12, and 14, and three *epi*-sphaeropsidone derivatives, 11, 13, and 15, tested in the range 0.01–100  $\mu\text{M}$ . \*Indicates differences at the 0.05 level compared with the control.

each compound, decreased concentrations did not induce *S. hermonthica* haustorium while higher concentrations showed inhibition activity of *S. hermonthica* germination (Figure 4).

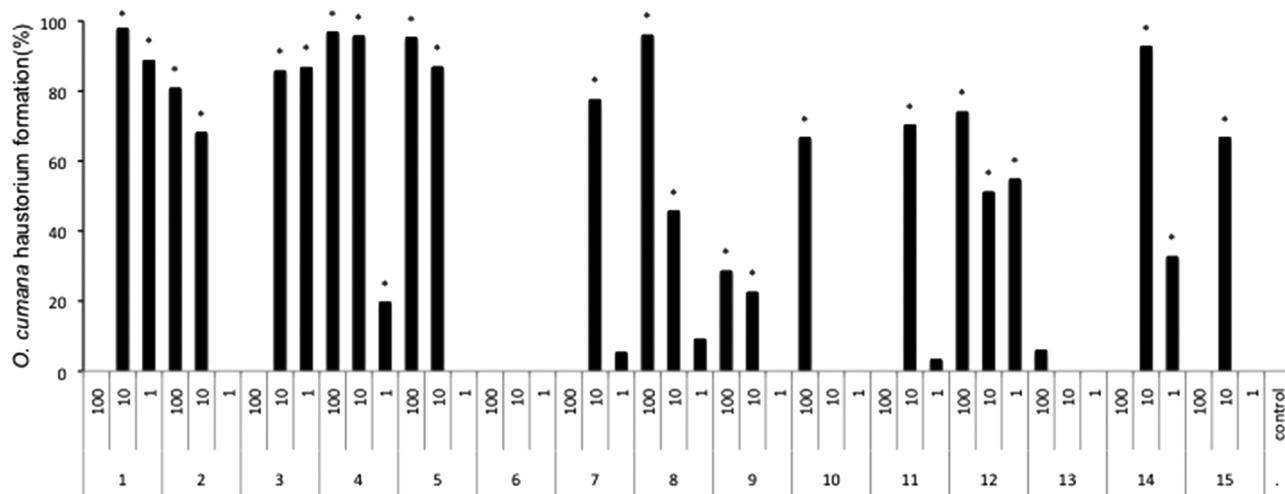
Necrosis was not detected in *S. hermonthica* radicles during the length of the experiment at any concentration. Both phytotoxic and organogenic activities in radicles of parasitic weeds has been previously observed for haustorium-inducing factors.<sup>31</sup> Both active concentrations of sphaeropsidone, 1 and 0.1  $\mu\text{M}$ , induced similar rate of haustorium induction (85% of radicles developed haustorium) (Figure 3). However, the radicles grew longer before detecting the haustorium-inducing signal at 0.1 than at 1  $\mu\text{M}$ , visualized by longer radicles terminated in haustorium (data not shown). The same phenomenon was observed with *epi*-sphaeropsidone as *S. hermonthica* radicles terminated in haustorium were longer when treated at 1  $\mu\text{M}$  than at 10  $\mu\text{M}$ . This could be indicating that the required time of exposure for signal detection and haustorium development was indirectly related with the compound concentration. Parasitic radicle responding later to decreased concentrations of haustorium-inducing signal could constitute a mechanism aimed to measure distance for host attachment.<sup>30</sup> Several phenolic derivatives of plant origin have been previously identified as haustorium-inducing factors for *Striga*.<sup>4,15,30</sup> Many of those phytochemicals are commonly

found in the rhizosphere and play roles in chemical communication with microbial organisms. Fungal phytotoxins have been described previously with potential to trigger host-dependent developmental programs in parasitic plants such as germination<sup>20,21</sup> but never were related before to the chemistry of haustorium initiation. Active concentrations of 1 and 2 where similar to those performed by previously known haustorium-inducing factors such as xenognosin A and B, 2,6-dimethoxybenzoquinone, ferulic, vanillic, sinapic, and syringic acids.<sup>15,30,32</sup>

Radicles of *O. crenata* and *O. cumana* responded to sphaeropsidone and *epi*-sphaeropsidone with a cessation of radicle elongation, the radicle tip became spherical, and crowns of cell extensions in the form of papillae were observed at the outer surface around the apex (Figure 2D,F). This observation is in agreement with the description of anchoring device of *Orobanchae* haustoria made by Joel and Losner-Goshen (1994).<sup>8</sup> According with *S. hermonthica* results, *epi*-sphaeropsidone was a weaker *Orobanchae* haustorium-inducer than sphaeropsidone. These results may suggest that the haustorial activity of sphaeropsidones is mediated by a similar mechanism in *Striga* and *Orobanchae*. The activity of both sphaeropsidones in *Orobanchae* radicles was slightly weaker than that observed in *Striga* radicles. Haustorium was observed in *Orobanchae* radicles 364



**Figure 5.** Induction of *O. crenata* haustorium formation by sphaeropsidone, **1**, *epi*-sphaeropsidone, **2**, two natural analogues, **3** and **4**, eight sphaeropsidones derivatives, **5–10**, **12**, and **14**, and three *epi*-sphaeropsidone derivatives, **11**, **13**, and **15**, tested in the range 1–100 μM. \*Indicates differences at the 0.05 level compared with the control.



**Figure 6.** Induction of *O. cumana* haustorium formation by sphaeropsidone, **1**, *epi*-sphaeropsidone, **2**, two natural analogues, **3** and **4**, eight sphaeropsidones derivatives, **5–10**, **12**, and **14**, and three *epi*-sphaeropsidone derivatives, **11**, **13**, and **15**, tested in the range 1–100 μM. \*Indicates differences at the 0.05 level compared with the control.

365 treated with sphaeropsidone at concentrations between 10 and  
 366 1 μM and with *epi*-sphaeropsidone at concentrations between  
 367 100 and 10 μM (Figures 5 and 6). The induction of haustorium  
 368 development by exogenous signals has never been documented  
 369 for *Orobanchae*, and therefore our results constitute a break-  
 370 through against the general belief that *Orobanchae* does not  
 371 respond to exogenous stimulation in order to develop  
 372 haustoria.<sup>8–10</sup> Sphaeropsidone at 100 μM did not inhibit  
 373 *Orobanchae* germination but it was phytotoxic, inhibiting  
 374 *Orobanchae* radicle development leading to haustorium inhibi-  
 375 tion (Figures 5 and 6). The inhibitory activity developed by  
 376 sphaeropsidones at high concentrations differentially targeted  
 377 the processes of germination in *Striga* and radicle development  
 378 in *Orobanchae*. Phytotoxicity developed by haustorium inducing  
 379 factors has been previously associated with a common  
 380 metabolic pathway shared by germination and root growth  
 381 processes.<sup>5</sup>

382 These results prompted to prepare some derivatives of both  
 383 **1** and **2** to elucidate patterns in the molecular specificity of  
 384 haustorial induction in *S. hermonthica*, *O. crenata*, and *O.*  
 385 *cumana*. *epi*-Sphaeropsidone, **2** (Figure 1), is a natural isomer of

**1** (Figure 1), being its epimer at C-5, and the chlorosphaer-  
 opsidones **3** and **4** (Figure 1) are two C-6 epimeric natural  
 analogues of sphaeropsidones showing the opening of the  
 epoxy ring and its conversion in the corresponding chloridrin,  
 with the chlorine at C-6 and the hydroxy group at C-5.

Six already known derivatives of sphaeropsidone (**5–10**,  
 Scheme 1) and one of *epi*-sphaeropsidone (**11**, Scheme 2) were  
 hemisynthesized and showed the same spectroscopic properties  
 previously reported.<sup>26</sup> Derivative **5** showed the reversible  
 acetylation of the hydroxy group at C-5, while the 2,4,5-  
 acetoxyanisole, **6**, obtained from the same acetylation reaction,  
 is a tetrasubstituted benzene derivative and thus also the  
 aromatization of the cyclohexenone ring. The same hydroxy  
 group at C-5 was converted by oxidation to carbonyl group,  
 yielding the corresponding 2,3-epoxyhemiquinone **7**, while the  
 reduction of the carbonyl group at C-2 generated the  
 corresponding 3-methoxy-cyclohex-3-ene-2,5-diol **9**. **1** was  
 also converted into the corresponding bromydrin **8**, which  
 differs from the epichlorosphaeropsidone **4** for the halogen at  
 C-1, which has a bromine instead of chlorine, and for the  
 opposite stereochemistry of the hydroxy group at C-5. The

407 catalytic hydrogenation of **1** yielded 2,4-dihydroxy-5-methox-  
408 cyclohexanone **10**, which showed the saturation of the double  
409 bond between C-2 and C-3 and the reductive opening of the  
410 epoxy ring. *epi*-Sphaeropsidone, **2**, was converted into the  
411 corresponding acetyl derivative **11**, which, although epimer at  
412 C-5, showed as **5** the reversible esterification of the hydroxy  
413 group at the same carbon.

414 Sphaeropsidone, **1** was converted into the new esters **12** and  
415 **14** (Scheme 1), which showed always the reversible  
416 modification of the hydroxy group at C-5 although the acyl  
417 group is different, being a *p*-Br-benzoyl in **12** and 5-  
418 azidopentanoyl in **14**. Both derivatives **12** and **14** showed  
419 spectroscopic properties consistent with their structures.  
420 Significantly, their IR spectra did not show bands for hydroxy  
421 groups.<sup>33</sup> In particular, the <sup>1</sup>H NMR spectrum of **12** differed  
422 from that of **1** for the expected downfield shift ( $\Delta\delta$  0.96) of H-  
423 **5** appearing as a doublet ( $J = 2.5$  Hz) and for the couple of two  
424 doublets ( $J = 8.7$  Hz) of the protons of the *para*-substituted  
425 benzene ring resonating at  $\delta$  8.01 and 7.63 for H-2',6' and H-  
426 3',5', respectively. Its ESIMS spectrum showed the character-  
427 istic isotopic peaks of bromine for both sodium clusters  $[M + 2$   
428  $+ Na]^+$  and  $[M + Na]^+$  and the pseudomolecular ion  $[M + 2 +$   
429  $H]^+$  and  $[M + H]^+$  at  $m/z$  364 and 362, and 342 and 340,  
430 respectively. The <sup>1</sup>H NMR spectrum of **14** differed from that of  
431 **1** for the expected downfield shift ( $\Delta\delta$  0.74) of H-5, which  
432 resonated as a doublet ( $J = 2.9$  Hz) at  $\delta$  5.97, and for the  
433 presence of the signals of the 5-azidopentanoyl group appearing  
434 as two triplets ( $J = 6.5$  and 7.3 Hz, respectively) at  $\delta$  3.32 and  
435 2.53 for the methylene protons, respectively, linked to the azido  
436 and the carboxyl group. The other two methylene groups  
437 appeared as multiplets at 1.83–1.76 (H<sub>2</sub>C-3') and 1.71–1.64  
438 (H<sub>2</sub>C-4'). Its ESIMS spectrum showed the sodium cluster  $[M$   
439  $+ Na]^+$  and the pseudomolecular ion  $[M + H]^+$  at  $m/z$  304 and  
440 282, respectively.

441 *epi*-Sphaeropsidone, **2**, was chemically reduced into the  
442 corresponding 3-methoxy-cyclohex-3-ene-2,5-diol, **13** (Scheme  
443 **2**), which is an epimer at C-5 of **9**, similarly obtained from **1**.  
444 The <sup>1</sup>H NMR spectrum of **13** differed from that of **2** for the  
445 significant presence of the broad doublet ( $J = 10.4$  Hz) of H-2  
446 and for the expected upfield shift ( $\Delta\delta$  0.66) of H-3, which  
447 appeared as a broad singlet at  $\delta$  4.57 instead of a doublet. Its  
448 ESIMS spectrum showed the sodium cluster  $[M + Na]^+$  and  
449 the pseudomolecular ion  $[M + H]^+$  at  $m/z$  182 and 159,  
450 respectively. As **1** was converted into **14**, **2** was similarly  
451 esterified into the corresponding 5-azidopentanoyl derivative  
452 **15** (Scheme 2). Its IR spectrum did not show a band for a  
453 hydroxy group. The <sup>1</sup>H NMR spectrum of **15** differed from that  
454 of **2** for the expected downfield shift ( $\Delta\delta$  1.28) of H-5, which  
455 appeared as a broad singlet at  $\delta$  5.92 and for the presence of the  
456 signals of the 5-azidopentanoyl group appearing as two triplets  
457 ( $J = 6.5$  and 7.2 Hz, respectively) at  $\delta$  3.31 and 2.45 for the  
458 methylene protons, respectively, linked to the azido and the  
459 carboxyl group. The other two methylene protons appeared  
460 both as multiplets at  $\delta$  1.80–1.72 (H<sub>2</sub>C-3') and 1.71–1.68  
461 (H<sub>2</sub>C-4'). Its ESIMS spectrum showed the sodium cluster  $[M$   
462  $+ Na]^+$  and the pseudomolecular ion  $[M + H]^+$  at  $m/z$  304 and  
463 282, respectively.

464 The haustorium-inducing activity in *S. hermonthica* of  
465 sphaeropsidones (**1**–**4**) and their derivatives (**5**–**15**) is  
466 shown in Figure 3. The *S. hermonthica* assays indicated that  
467 conversion of **1** in derivatives **6** and **10** led to a severe reduction  
468 in the haustorium-inducing activity being only significant at 100  
469  $\mu$ M, while conversion in derivatives **4**, **7**, **8**, and **9** led to loss at

0.1  $\mu$ M of haustorium-inducing activity that characterizes the  
parent compound. Structural modifications in **3**, **5**, **12**, and **14**  
did not affect the haustorial activity, inducing high rates of *S.*  
*hermonthica* haustoria at 1 and 0.1  $\mu$ M comparable with  
sphaeropsidone, **1**.

Esterification at C-5 in **11** and **15** increased haustorium-  
inducing activity on *S. hermonthica* seedlings in comparison  
with **2**, and in derivative **14** in comparison with **1**, while the  
modifications at C-5 made in **3** and **12** maintained the strong  
activity observed in **1** up to 0.1  $\mu$ M. Acetylation of **1** in  
derivatives **5** did not strongly affect the haustorial activity as **1**  
and **5** are strong inducers, although a slight reduction in activity  
was found at 0.1  $\mu$ M in derivative **5** in comparison with **1**. It is  
interesting to note that **2** and derivative **11** are weaker  
haustorial inducers when compared with their respective  
epimers of **1** and **5**. These results allow us to hypothesize  
that the stereochemistry at C-5, although not strongly, play an  
important role in impart this activity.

The epoxy ring by itself was not essentially associated with  
haustorial inducing activity as some derivatives lacking the  
epoxy ring, i.e., the analogue **3**, had strong haustorial activity,  
however, when the selective reductive opening of the epoxy  
ring was associated with the reduction of the olefinic double  
bond in **10** or with the aromatization of the cyclohexenone ring  
in **6**, the haustorial-inducing activity was strongly affected. The  
reduction of the C-2 carbonyl group in **9** and **13** led to  
reduction of haustorial activity in comparison with their parent  
compounds **1** and **2** in seedlings.

These results could be explained, except for **7**, in the easy  
conversion of all derivatives at physiological pH into  
sphaeropsidone and then into the corresponding above  
hypothesized 2,3-epoxy-5-methoxyquinone. In fact, **3**, having  
the requested stereochemistry of the hydroxy and chlorine  
groups at C-5 and C-6, according to the well-known  
intramolecular nucleophilic substitution could give **1**. This  
reaction is less easy for the other chloridrin **4**, probably for the  
opposite not suitable stereochemistry of chlorine at C-6.<sup>34</sup>  
Furthermore, derivatives **5**, **12**, and **14** being ester of **1** at  
physiological pH, according to the well-known lethal  
metabolism,<sup>35</sup> probably were hydrolyzed into **1**. The slight  
minor activity of derivative **8** is due to the same reasons above-  
reported for **4** having the bromine in **8** the same stereo-  
chemistry of chlorine in **4**. The reduced activity of **9** could be  
explained from different oxidation stage in respect to **1** being **9**,  
a 3-methoxy-cyclohex-3-ene-2,5-diol, and **1**, a 2,3-epoxy-5-  
methoxyhemiquinone. The noteworthy reduction of the activity  
of derivatives **6** and **10** was due to the inability to convert their  
benzene and the cyclohexanone rings into 2,3-epoxyhemiqui-  
none ring of sphaeropsidone and then in the corresponding  
2,3-epoxy-5-methoxyquinone.

The haustorium-inducing activity in *O. crenata* and *O.*  
*cumana* of sphaeropsidones (**1**–**4**) and their derivatives (**5**–  
**15**), is shown in Figures 5 and 6, respectively. SAR results  
indicated the haustorium-inducing activity of the parent  
compound was affected by the structural modifications in a  
slightly different way for *Striga* and *Orobanch* species, being  
those differences more obvious between *Striga* and *O. cumana*.  
The reduction of C-2 carbonyl group in **9** and **13** led to  
reduction of haustorial activity at 1  $\mu$ M in *O. crenata* seedlings,  
but it induced a strong loss of activity at all concentrations in *O.*  
*cumana*. A marked loss of haustorial-inducing activity was  
observed in *O. crenata* for compounds **6** and **10** when  
compared with **1**, as it was observed for *S. hermonthica*, while

the activity was completely lost for *O. cumana*. For *O. crenata*, structural modifications in compounds **5** and **12** maintained nearly the same levels of haustorial activity than **1**, as it happened for *S. hermonthica*, however, activity of compound **5** was lost at 1  $\mu\text{M}$  for *O. cumana*. The activity of compounds **4** and **8** was observed to be very high for *O. crenata*, being the haustorial papillae especially well developed by compound **4** in both *Orobanche* species. Differential effects caused by structural modifications across hemiparasitic genera have been previously suggested. Steffens et al. (1982)<sup>32</sup> found that for the system *Agalinis purpurea*–xenognosin A, haustorium was initiated when the molecular inducer meet two structural features: (i) hydroxy and methoxy groups were present and both groups occupying a *meta*-position and (ii) an alkyl branching *ortho* to the methoxy substituent. MacQueen (1984)<sup>15</sup> studied the activity of phenolic compounds on *S. hermonthica* and demonstrated that the activity was also initiated when the hydroxy group was occupying a *para*-position and the methoxy group a *meta*-position while the alkyl branching *ortho* to the methoxy substituent was not required for activity in *Striga*.

Regarding germination inhibition, the activity of the two natural analogues (**3** and **4**), eight sphaeropsidones derivatives (**5**–**10**, **12**, and **14**), and three *epi*-sphaeropsidone derivatives (**11**, **13**, and **15**) in comparison with sphaeropsidone, **1**, and *epi*-sphaeropsidone, **2**, was tested on *S. hermonthica* (Figure 4). The germination inhibitory activity was reduced in **6**, **8**, **9**, and **10** and increased in **5**, **7**, **12**, and **14** in comparison with sphaeropsidone, **1**, while it was reduced in **13** and increased in **11** and **15** in comparison with *epi*-sphaeropsidone, **2**. The increased germination inhibitory activity in **5**, **11**, **12**, **14**, and **15** can be explained by their increased lipophilicity which could facilitate cell membrane permeability.<sup>35</sup> The reduced germination inhibitory activity in **9** and **13** could be explained for the absence of the  $\alpha,\beta$ -unsaturated ketone due to the reduction of the carbonyl group at C-2, and in **6** and **10** for the aromatization of the cyclohexenone ring, and for the reduction of the double bond between C-4 and C-5 and for the reductive opening of the epoxy ring.

The SAR study results suggested that the ability to initiate haustorium development in *S. hermonthica* is probably due to the conversion of the natural sphaeropsidones, their analogues, and hemisynthetic derivatives in the corresponding 4-methoxyquinone. This conversion could occur more easily in the analogue **3** and derivatives **5**, **8**, **12**, and **14** of sphaeropsidone and derivatives **11** and **15** of *epi*-sphaeropsidones. In fact, as above cited the esters **5**, **11**, **12**, and **14**, by hydrolysis, could be converted into the parent compounds as well as the analogue **3** and derivative **8**, by nucleophilic elimination.<sup>36</sup> A successive oxidation step could be converted both **1** and **2** into the 2,3-epoxy-4-methoxyquinone, and this finally, by reductive opening of the epoxy group followed by water nucleophilic elimination into the 4-methoxyquinone. This hypothesis on the mode of action of sphaeropsidone is in full agreement with the results obtained using natural and synthetic quinones as sorghum xenognosin and dimethoxybenzoquinones, the latter very closely related to the 4-methoxyquinone, which as above explained could be generated by oxidation of sphaeropsidones in the studies carried out on haustoria and the chemistry in host recognition (ion) parasitic angiosperms. Quinone/hydroquinone structures serve as cofactors in many metabolic pathways, playing critical chemical roles in oxidation/reduction processes.<sup>31,36</sup> This mode of action could also operate in the haustorium-induction in *Orobanche*.

The slight difference observed between *Orobanche* and *Orobanche* is not surprising as differences between the species in their ability to recognize and respond to the same haustorium-inducing factors has been reported.<sup>31</sup> The presence of the epoxy ring and the  $\alpha,\beta$ -unsaturated ketone group could be the responsible for the inhibition of *S. hermonthica* seed germination.

The induction of haustorium is a little researched area in parasitic weeds, especially in *Orobanche* species. Our work has identified for the first time natural occurring compounds with haustorium-inducing activity for *Orobanche* species, which opens several research directions aimed either to understand the chemistry of haustorium initiation or toward the development of alternatives to conventional control. It is now possible to envision research in which host-encoded haustorium-inducing factors are identified through bioactivity-guided chromatographic purification of host root extracts. In addition, *Orobanche* transgenic seedlings in which candidate genes for haustorium initiation are silenced could be easily phenotyped by the in vitro application of haustorium inducers. It could be possible, in addition, once host-encoded haustorium factors are identified for *Orobanche*, to select crop cultivars with low haustorium-induction activity in their roots. In addition, this work also opens the way of the development of new agrochemicals with a novel mode of action based on disturbing the fine-tuned process of host recognition. Compounds such as sphaeropsidones with the ability of triggering haustorium induction in a suicidal fashion are good candidates for such strategy. Current research in our laboratory is starting to explore the above-mentioned alternatives for *Orobanche* weed management.

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### Notes

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