Phytopathol. Mediterr. (2000) 39, 299-309

Chlorosphaeropsidone and epichlorosphaeropsidone, two new chlorinated dimedone methyl ethers isolated from liquid cultures of *Sphaeropsis sapinea* f. sp. *cupressi*

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Summary. Two new dimedone methyl ethers, named chlorosphaeropsidone and epichlorosphaeropsidone were isolated from the culture filtrates of *Sphaeropsis sapinea* f. sp. *cupressi*, the causal agent of a canker of Italian cypress (*Cupressus sempervirens* L.). The same fungus also produced various phytotoxic metabolites: three pimarane diterpenes (sphaeropsidins A, B and C) and two dimedone methyl ethers (sphaeropsidone and episphaeropsidone). Chlorosphaeropsidone and epichlorosphaeropsidone were characterized using chemical and spectroscopic methods as two 6-chloro-4,5-dihydroxy-3-methoxycyclohex-2-en-1-ones epimers at C-6. Their relative and absolute configurations were established by NMR, X-ray analysis and circular dichroism. Assayed on host plants they did not show phytotoxic activity. Comparison with other sphaeropsidones suggested that this might result from opening of the epoxy ring.

Key words: *Sphaeropsis sapinea* f. sp. *cupressi*, cypress canker disease, dimedone methyl ethers, chlorosphaeropsidone, epichlorosphaeropsidone.

Introduction

Several fungi, including species of *Seiridium*, *Sphaeropsis* and *Botryosphaeria*, produce cankers on wild and cultivated species of *Cupressus* and related conifers. *Sphaeropsis sapinea* f. sp. *cupressi* is a fungal pathogen which causes cankers on the stems and branches of Italian cypress trees (*Cupressus sempervirens* L.) (Fig. 1) (Frisullo *et al.*, 1997) cultivated in the Mediterranean region. These cankers differ from the epidemic cypress blight caused by *Seiridium* spp. which has already devastated cypress trees in the Mediterranean area (Graniti, 1998).

From the ethyl acetate extract of the *in vitro* culture filtrates of *S. sapinea* f. sp. *cupressi* we isolated and characterized five phytotoxic metabolites: three pimarane diterpenes, the sphaeropsidins A, B and C (Evidente *et al.*, 1996; 1997) and two dimedone methyl ethers, named sphaeropsidone and episphaeropsidone (**5** and **6**, in Fig. 2) (Evidente *et al.*, 1998).

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Fig. 1. Yellowing, browning and necrosis of branches (left) and stem canker (right) caused by *Sphaeropsis sapinea* f. sp. *cupressi* on *Cupressus sempervirens*.

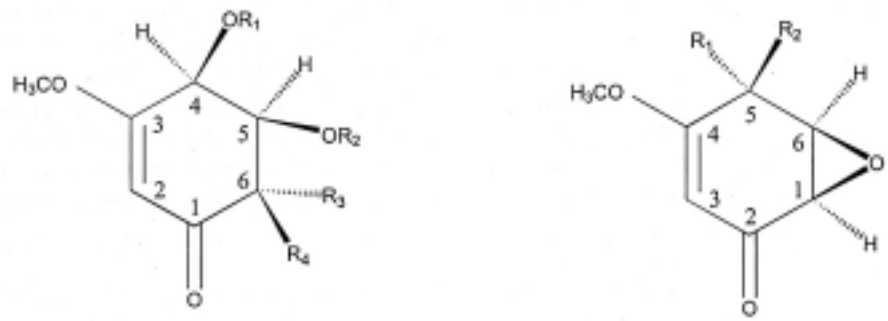
Organic extracts of culture filtrates of the above fungus contained at least two other, more polar metabolites, present at very low concentrations. These were found to be two new dimedone methyl ethers, structurally related to sphaeropsidones (**5** and **6**).

This paper describes the isolation, the chemical characterization and the biological investigation of these new dimedone methyl ethers, chlorosphaeropsidone and epichlorosphaeropsidone (**1** and **2**, Fig 2), respectively. Their structure and configuration were confirmed by X-ray analysis while the absolute stereochemistry was deduced by circular dichroism and NMR.

Materials and methods

General experimental procedure

Optical rotations were measured in methanol on a JASCO DIP-370 Polarimeter (Jasco, Tokyo, Japan). The IR spectra were determined in chloroform on a Perkin-Elmer IR FT-1720X spectrometer (Perkin-Elmer, Norwalk, CT, USA), the UV spectra in methanol on a Perkin-Elmer Lamda 3B spectrophotometer. CD spectra were recorded in methanol on a JASCO J-715 spectropolarimeter. ^{1}H NMR spectra were recorded at 500 and at 250 MHz in $CDCl_3$, ^{13}C NMR spectra at 125 and at 62.5 MHz in $CDCl_3$, on DRX 500 and AM 250 Bruker spectrometers (Bruker, Karlsruhe, Germany). The same solvent was used as internal standard. Carbon multiplicities were determined by DEPT spectra (Breitmaier and Voelter, 1987). DEPT, COSY-45 and HMQC NMR experiments were performed using Bruker microprograms. EI- and HR EI- and FAB-MS were taken at 70 eV and in glycerol/thioglycerol, respectively, on a Fisons Trio-2000 (VG Instruments, Manchester, UK) and a Fisons ProSpec spectrometer. Analytical and preparative TLC were performed on silica gel plates (Kieselgel 60 F_{254}, 0.25 and 0.50 mm, re-



1 R_1=R_2=R_4=H, R_3=Cl

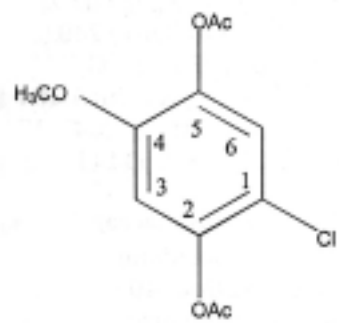
2 R_1=R_2=R_3=H, R_4=Cl

3 R_1=R_2=Ac, R_3=Cl, R_4=H

4 R_1=R_2=R_4=H, R_3=Br

5 R_1=H, R_2=OH

6 R_1=OH, R_2=H



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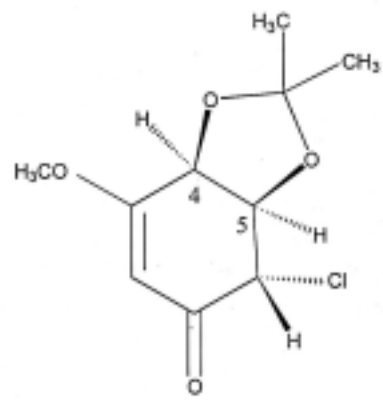

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Fig. 2. Structure formulae of: chlorosphaeropsidone (**1**), its derivatives (**3**,**7** and **8**), epichlorosphaeropsidone (**2**), sphaeropsidone and its bromohydrin (**5** and **4**) and episphaeropsidone (**6**).

spectively, Merck, Darmstadt, Germany); the spots were visualized by exposure to UV radiation and/or by spraying with 10% H_2SO_4 in methanol and then with 5% phosphomolybdic acid in methanol, followed by heating to 110°C for 10 min. Column chromatography was performed on silica gel (Merck, Kieselgel 60, 0.063-0.20 mm).

Fungal strain, culture medium and growth conditions

Sphaeropsis sapinea f. sp. *cupressi* was isolated from the cortical tissue of infected cypress (*Cupressus sempervirens*) trees collected in Morocco and in Italy. A single conidium isolate of *S. sapinea* f. sp. *cupressi* (Italian isolate) was grown in Petri dishes containing potato-sucrose (2%)-agar medium (PSA) and then transferred to slants containing the same medium, at 25°C for 2 weeks. The subcultures were stored at 5°C in the fungal collection of the Dipartimento di Biologia e Patologia Vegetale, Università di Bari, Italy (N. 1524).

Production, extraction and purification of chlorosphaeropsidones

The culture filtrates (10 l) of *S. sapinea* f. sp. *cupressi*, obtained as described in earlier study (Evidente *et al.*, 1996), were acidified to pH 4 with 2 M HCl and extracted with ethyl acetate (4x5 l). The combined organic extracts were dried (Na_2SO_4) and evaporated under reduced pressure to give a red-brown oily residue (2.84 g) having high phytotoxic activity. TLC analysis of this residue on silica gel (eluent chloroform:*iso*-propanol, 95:5) showed the presence of sphaeropsidins A, B and C (R_f 0.65, 0.43 and 0.50 respectively), sphaeropsidone and episphaeropsidone (R_f 0.38) and that of at least two others, more polar metabolites (R_f 0.21 and 0.14, respectively). The crude oily residue was chromatographed on a silica gel column eluted with chloroform:*iso*-propanol (95:5) giving 9 groups of homogeneous fraction, in earlier studies (Evidente *et al.*, 1996; 1998). From purification of groups 6 and 7 by crystallisation, and the combination of column chromatography and preparative TLC, the pure sphaeropsidins A, B and C (681.9, 150, and 62.4 mg, respectively) and sphaeropsidone and episphaeropsidone (44.0 and 19.7 mg, respectively) were obtained. The residue (234.0 mg) of fraction 8 containing the two more polar metabolites (R_f 0.21 and 0.14) was purified by preparative TLC on silica gel (eluent chloroform:*iso*-propanol, 9:1) giving two crude metabolites (74.0 and 31.1 mg, respectively). These were definitely purified by two further steps of preparative TLC on silica gel (eluents petroleum ether-acetone, 7:3 and 6:4), giving chlorosphaeropsidone and epichlorosphaeropsidone (**1** and **2**, 44.0 and 19.7 mg, 4.4 and 2.0 mg l^{-1} respectively) as homogeneous oils, as shown by their TLC behaviour, in different systems. TLC of **1** and **2** on silica gel (eluents chloroform:*iso*-propanol, 9:1 and petroleum ether-acetone, 1:1) and on reverse phase (eluent ethyl alcohol-H_2O, 45:55) showed respectively a R_f of 0.40 and 0.53; 0.67 and 0.26; 0.34 and 0.75.

Chlorosphaeropsidone (**1**, Fig. 2)

$[\alpha]^{25}D$ +73.9 (c 0.4); CD λ_{max} nm ($[\phi]^{25}$) 248 (+136222), 302 (-935); UV λ_{max} nm (log ε) 249 nm (3.93); IR: ν_{max} 3460, 1677, 1603 cm^{-1}; ^1H and ^{13}C NMR see Table 1. EI-MS *m/z* (rel. int.) 194 $[M+2]^+$ (1), 192 $[M]^+$ (3), 176 $[M+2-H_2O]^+$ (8), 174 $[M-H_2O]^+$ (24), 139 $[M-H_2O-Cl]^+$ (48), 58 (100). HR EI-MS *m/z* 174.0042 (calcd for $C_7H_7ClO_3$, 174.0084); HR FAB-MS *m/z* 193.0228 (calcd for $C_7H_{10}ClO_4$, 193.0268) $[M+H]^+$.

Epichlorosphaeropsidone (**2**, Fig. 2)

$[\alpha]^{25}D$ +53.7 (c 0.3); CD λ_{max} nm ($[\phi]^{25}$) 260 (+2112); UV λ_{max} nm (log ε) 249 (3.91); IR ν_{max} 3463, 1677, 1603 cm^{-1}; ^1H and ^{13}C NMR spectra see Table 1. EI-MS, *m/z* (rel. int.) 194 $[M+2]^+$ (2), 192 $[M]^+$ (6), 176 $[M+2-H_2O]^+$ (34), 174 $[M-H_2O]^+$ (87), 139 $[M+H_2O-Cl]^+$ (96), 114 (100).

Acetylation of chorosphaeropsidone

Chlorosphaeropsidone (**1**, 4.0 mg) was acetylated with dry pyridine (40 µl) and acetic anhydride (40 µl) at room temperature. Overnight the reaction was stopped by addition of MeOH and the pyridine was eliminated by azeotrope with C_6H_6.

Table 1. ^1H and ^{13}C NMR spectral data of chlorosphaeropsidone and epichlorosphaeropsidone (**1** and **2**)[a].

	1[b]			**2**[b]		
C	δ_C[c]	δ_H	*J* (Hz)	δ_C[c]	δ_H	*J* (Hz)
1	190.5 *s*	-	-	188.7 *s*	-	-
2	100.6 *d*	5.46 *br s*	-	100.0 *d*	5.45 *br d*	1.2
3	174.5 *s*	-	-	170.9 *s*	-	-
4	67.9 *d*	4.55 *d*	3.7	69.0 *d*	4.55 *d*	3.7
5	72.1 *d*	4.14 *dd*	8.2, 3.7	73.2 *d*	4.44 *dd*	3.7, 2.9
6	60.8 *d*	4.60 *d*	8.2	63.6 *d*	4.53 *dd*	2.9, 1.2
OMe	56.5 *q*	3.79 *s*		56.6 *q*	3.76 *s*	

[a] The chemical shifts are in δ values (ppm) from TMS.
[b] 2D ^1H-^1H (COSY) and 2D ^{13}C-^1H (HMQC) NMR experiments delineated the correlations of all protons and the corresponding carbons.
[c] Multiplicities determined by DEPT spectra.

The residue showed by TLC analysis on silica gel (eluent chloroform:*iso*-propanol, 97:3) the presence of two derivatives having R*f* 0.81 and 0.67 (UV-absorbing) respectively. The mixture was purified by preparative TLC under the same conditions, giving the 4,5-*O,O'*-diacetyl derivative **3** (1.0 mg) and the aromatized derivative 2,5-diacetoxy-4-methoxychlorobenzene **7** (1.4 mg), both as homogeneous oils. Derivative **3**: IR ν_{max} 1753, 1626 cm^{-1}; ^1H NMR δ 6.01(1H, *d*, *J*=3.7 Hz, H-4), 5.58 (1H, *br s*, H-2), 5.42 (1H, *dd*, *J*=3.7 and 9.1 Hz, H-5), 4.56 (1H, *d*, *J*=9.1 Hz, H-6), 3.78 (3H, *s*, OMe), 2.15 (3H, *s*, MeCO), 2.09 (3H, *s*, MeCO). EI-MS *m/z* (rel. int.) 236 $[M+2\text{-}CH_2CO]^+$ (2), 234 $[M\text{-}CH_2CO]^+$ (6), 218 $[M+2\text{-}AcOH]^+$ (6), 216 $[M\text{-}AcOH]^+$ (16), 176 $[M+2\text{-}CH_2CO\text{-}AcOH]^+$ (33), 174 $[M\text{-}CH_2CO\text{-}AcOH]^+$ (100), 161 $[M+2\text{-}CH_2CO\text{-}AcOH\text{-}Me]^+$ (23), 159 $[M\text{-}CH_2CO\text{-}AcOH\text{-}Me]^+$ (68), 139 $[M\text{-}CH_2CO\text{-}AcOH\text{-}Cl]^+$ (17). Derivative **7**: UV λ_{max} nm (log ε) 280 (3.22); IR $\nu_{max}\psi$1766, 1644, 1505 cm^{-1}; ^1H NMR δ 6.75 and 6.65 (1H each, *s* and *s*, H-3 and H-6), 3.80 (3H, *s*, OMe), 2.34 (*s*, 3H, MeCO), 2.30 (*s*, 3H, MeCO). EI-MS *m/z* (rel. int.) 260 $[M+2]^+$ (8), 258 $[M]^+$ (27), 218 $[M+2\text{-}CH_2CO]^+$ (19), 216 $[M\text{-}CH_2CO]^+$ (58), 176 $[M+2\text{-}2x CH_2CO]^+$ (33), 174 $[M+2\text{-}2x CH_2CO]^+$ (100), 161 $[M+2\text{-}2x CH_2CO\text{-}Me]^+$ (34), 159 $[M\text{-}2x CH_2CO\text{-}Me]^+$ (100).

4,5-*O,O'*-Isopropylidene derivative of chlorosphaeropsidone

Chlorosphaeropsidone (**1**, 14 mg) was dissolved in 2,2-dimethoxypropane (0.5 ml) and treated with two drops of concentrated HCl for 2 h at room temperature under stirring. The reaction was dried by a N_2 stream and the residue purified by preparative TLC on silica gel, (eluent chloroform:*iso*-propanol, 95:5) to give the 4,5-*O,O'*-isopropylidene derivative **8** (1.8 mg) as a homogeneous oil: UV λ_{max} nm (log ε) 244 (3.91); IR ν_{max} 1687, 1620 cm^{-1}; ^1H NMR δ 5.51 (1H, *br s*, H-2), 5.46 (1H, *d*, *J*=8.5 Hz, H-6), 4.78 (1H, *d*, *J*=6.3 Hz, H-4), 4.54 (1H, *dd*, *J*=8.5 and 6.3 Hz, H-5) , 3.83 (3H, *s*, OMe), 1.57 and 1.46 (3H each, *s*, Me_2CO_2). EI-MS *m/z* (rel. int.) 234 $[M+2]^+$ (1), 232 $[M]^+$ (3), 219 $[M+2\text{-}Me]^+$ (2.5), 217 $[M\text{-}Me]^+$ (8), 190 $[M+2\text{-}CH_3CHO]^+$ (17), 188 $[M\text{-}CH_3CHO]^+$ (51), 176 $[M+2\text{-}Me_2CO]^+$ (25), 174 $[M\text{-}Me_2CO]^+$ (72), 158 $[M+2\text{-}Me_2CO\text{-}H_2O]^+$ (33), 156 $[M\text{-}Me_2CO\text{-}H_2O]^+$ (100), 139 $[M\text{-}Me_2CO\text{-}Cl]^+$ (96), 129 $[M+2\text{-}Me_2CO\text{-}H_2O\text{-}HCO]^+$ (33), 127 $[M+Me_2CO\text{-}H_2O\text{-}HCO]^+$ (98).

Conversion of chlorosphaeropsidone into the corresponding 6-epimer and sphaeropsidone

A solution of chlorosphaeropsidone (**1**, 1 mg) in 28 µl of 5% sodium bicarbonate and 20 µl methanol was allowed to stand for 10 min at room temperature. The reaction mixture was extracted with 8.0 ml ethyl acetate and extracts were washed with a small amount of water and then dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure to give 0.6 mg residue. This residue, when subjected to TLC analysis on silica gel (eluent chloroform:*iso*-propanol, 9:1), showed not only part of unreacted **1** but also the presence of two derivatives having R_f 0.47 and 0.26 respectively. They were co-chromatographed in two other TLC systems: silica gel (eluent petroleum ether-acetone, 6:4) and silica gel and reverse phase (eluent ethyl alcohol-H_2O, 45:55) with known samples of epichlorosphaeropsidone and sphaeropsidone (**2** and **5**).

Crystal data for 1

$C_7H_9ClO_4$, M=192.6, orthorhombic, space group $P\,2_12_12_1$, *a*=6.292(1), *b*=10.547(2), *c*=12.554(2) Å, V=833.1(4) $Å^3$, Z=4, T=293 K, D_{calcd} =1.53 g cm^{-3}, λ (Mo K_α) =0.7093 Å, µ =4.27 cm^{-1}, R(F) = 0.042 for 889 observed independent reflections (θ_{max} =26.5°) and 145 parameters. Data were collected on an MACH3 diffractometer using graphite-monochromated Mo K_α radiation (Entaf-Nonius, The Netherlands). The structure was solved by direct methods. The non-hydrogen atoms were refined anisotropically, the H-atoms isotropically. The refinement was by full-matrix (on *F*) least-squares with $w^{-1} = [\sigma^2(Fo) + (0.02\,Fo)^2 + 1]$.

Toxin bioassays

Culture filtrates, their chromatographic fractions and pure substances were assayed for phytotoxicity on three species of host (*Cupressus sempervirens* L. var. *pyramidalis*, *C. macrocarpa* Hart. var. lambertiana and *C. arizonica* Gr.) and non-host (tomato: *Lycopersicon esculentum* L. var. Marmande) as reported in an earlier study (Evidente *et al.*, 1998).

Results and discussion

Isolation of chlorosphaeropsidones

The crude oily residue (2.84 g) obtained by extraction of the culture filtrates (10 l) of *S. sapinea* f. sp. *cupressi* with ethyl acetate was fractionated

using a combination of column chromatography and preparative TLC to yield sphaeropsidin A, B, and C, sphaeropsidone (**5**), episphaeropsidone (**6**) and the two more polar metabolites (**1** and **2**) as shown by TLC on silica gel (eluents chloroform:*iso*-propanol, 95:5 and 9:1). The preliminary spectroscopic investigation on metabolites **1** and **2**, obtained as oily homogeneous compounds (4.4 and 2.0 mg l^{-1}), revealed that their structures were closely related to those of the sphaeropsidones. On the basis of the spectroscopic data shown below they were therefore named chlorosphaeropsidone and epichlorosphaeropsidone (**1** and **2**).

Biological assay of sphaeropsidones

When assayed on three species of Cupressus host (*C. sempervirens*, *C. macrocarpa* and *C. arizonica*) and on non-host tomato plants the two chlorosphaeropsidones (**1** and **2**) showed no sign of phytotoxicity in contrast with the significant symptoms induced by sphaeropsidone and episphaeropsidone (**5** and **6**) (Fig. 3).

Chemical characterization of the chlorosphaeropsidones

Chlorosphaeropsidone (**1**) had a molecular formula of $C_7H_9ClO_4$ as deduced from the HR FAB and EI-MS. Its IR spectrum showed bands characteristic of the hydroxy groups, of a carbonyl group and of the olefinic group of a vinylogous ester (Nakanishi and Solomon, 1977). This last structural feature was consistent with the absorption maximum of 249 nm observed in the UV spectrum, which was characteristic of β-methoxy-α,β-unsaturated cyclohexenone (Scott, 1964). The ^{1}H and the ^{13}C NMR spectra (Table 1) resembled those of sphaeropsidone (Evidente *et al.*, 1998) but with some marked differences. A broad singlet typical of an olefinic proton (H-2) appeared at δ 5.46, while a double doublet characteristic of an oxygenated secondary carbon (H-5) at δ 4.14 was coupled in the COSY spectrum (Bax and Freeman, 1981) with two doublets resonating at δ 4.60 and 4.55 (H-6 and H-4). In addition, a singlet of a methoxy group was observed at δ 3.79 (Pretsch *et al.*, 1983). As already

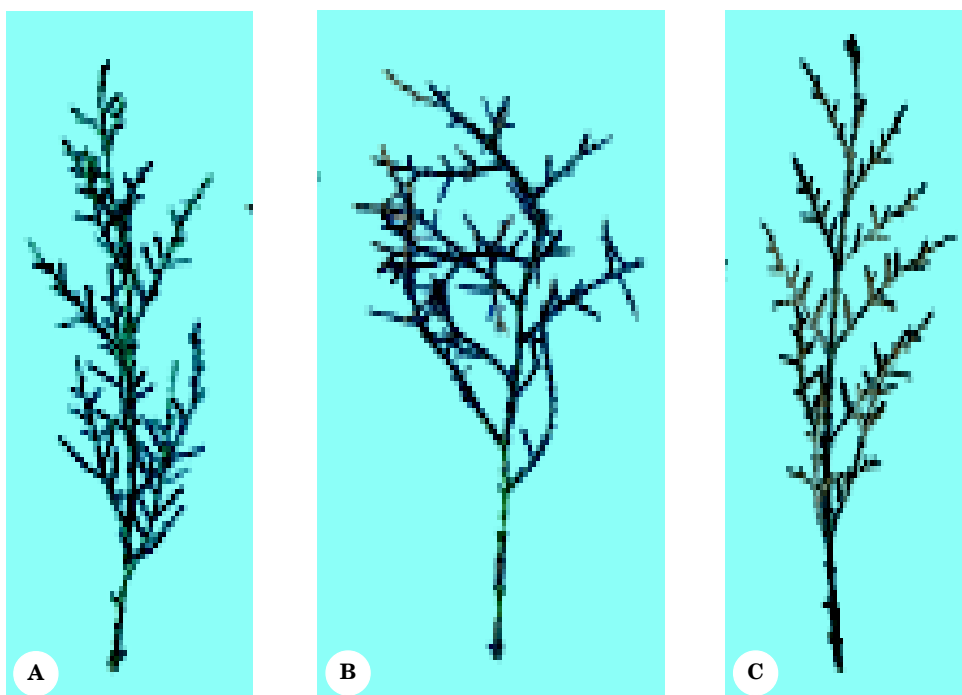

Fig. 3. Symptoms of browning and necrosis appeared on severed twigs of *Cupressus sempervirens* after treatment with 0.2 mg ml^{-1} solution of sphaeropsidone (B) and episphaeropsidone (C). Distilled water was used as a control (A).

mentioned for sphaeropsidone, the ^{13}C NMR spectrum showed signals characteristic of the β-methoxy-α,β-unsaturated cyclohexenone skeleton at δ 190.5, 174.5 and 100.6 for the carbonyl and quaternary methoxylated and protonated carbons of the trisubstituted double bond, C-1, C-3 and C-2 respectively, while the methoxy group appeared at δ 56.5 (Breitmaier and Voelter, 1987). A significant difference from sphaeropsidone was the absence of ^{1}H and ^{13}C NMR signals due to the oxirane ring, while the presence of a glycol system between C-4 and C-5 and a secondary chlorinated carbon (C-6) was noted. In the HMQC spectrum (Bax *et al*., 1990) H-4 and H-5 of the glycol system correlated with two secondary hydroxylated carbons at δ 67.9 and 72.1 (C-4 and C-5 respectively), while the secondary chlorinated carbon resonated at the typical chemical shift of δ 60.8 (Breitmaier and Voelter, 1987). Moreover, the glycol system proved to be *cis* since H-5 and H-6 were both axial-oriented, as indicated by their typical coupling constant of 8.2 Hz, and consequently H-4 was equatorial-orientated, as its coupling with H-5 was 3.7 Hz (Sternhell, 1969; Pretsch *et al*., 1983). The chlorine atom proved to be *trans*-oriented with respect to the hydroxy group at C-5, the latter being equatorial-oriented. Therefore chlorosphaeropsidone was assigned the structure **1** depicted in the Fig. 2. This structure was supported by the fragmentation peaks observed in the EI-MS spectrum. The molecular ion at *m/z* 192 by successive losses of H_2O and chlorine residue produced the ions at *m/z* 174.0042 ($C_7H_7ClO_3$) and 139; moreover, the characteristic isotopic ion at *m/z* 176 was generated by loss of H_2O from the Cl^{37} containing isotopic molecular ion (Pretsch *et al*., 1983). The HR FAB-MS showed a pseudomolecular ion $[M+H]^+$ at *m/z* 193.0228 ($C_7H_{10}ClO_4$).

Derivatization of the chlorosphaeropsidones (Fig. 2)

The structure was confirmed by acetylation carried out under standard conditions with pyridine and acetic anhydride, which yielded the corresponding 4,5-*O,O'*-diacetyl derivative **3** and the aromatized product **7**. The IR spectrum of **3** showed the absence of hydroxy groups and presence of bands typical of acetyl residues (Nakanishi and Solomon, 1977). Comparison of the ^{1}H NMR spectrum of **3** with that of **1** showed the only significant differences to be the downfield shifts (Δδ 1.46 and 1.28 ppm) of H-4 and H-5 at δ 6.01 and 5.42, and the presence of the acetyl singlets at δ 2.15 and 2.09. Finally, the EI-MS did not show the molecular ion but that significant at *m/z* 234 generated from it by loss of CH_2CO, together with the corresponding isotopic ion at *m/*z 236. Fragmentation ions due to the loss of the other acetyl and methyl groups and Cl residue were also observed, as reported in detail in the Materials and methods section. The aromatized derivative **7** showed the absence of hydroxy groups in the IR spectrum and the presence of acetyl groups (Nakanishi and Solomon, 1977), while the UV spectrum showed an absorption maximum at 280 nm, typical of a suitable substituted benzene derivative (Scott, 1964). Its ^{1}H NMR obviously was markedly different from that of **1** in which it showed only the singlets of the two para-coupled protons (H-3 and H-6) at δ 6.75 and 6.65, and singlets typical of a methoxy group and the two acetyl groups at δ 3.80, 2.34 and 2.30 respectively (Pretsch *et al*., 1983). Its EI-MS showed the molecular ion and the corresponding isotopic ion at *m/z* 258 and 260 respectively, together with fragmentation peaks due to the losses of the acetyl and methyl groups as reported in detail in the Materials and methods section.

The presence of a *cis*-glycol system in **1** was confirmed by preparing the corresponding isopropylidene derivative (**8**); this was done by bringing about an acid catalyzed reaction of chlorosphaeropsidone with 2,2-dimethoxypropane. The IR spectrum showed the absence of hydroxy groups. The ^{1}H NMR of the *cis*-glycol system protons differed from that of **1** in the downfield shifts (Δδ 0.23 and 0.40 ppm, respectively) of H-4 and H-5 at δ 4.78 and 4.54, together with the downfield shift (Δδ 0.86) of H-6 at δ 5.46, and in the presence of singlets of the two methyl of the isopropylidene group at δ 1.57 and 1.46. The EI-MS showed the molecular ion at *m/z* 232 and the corresponding isotopic peak at *m/z* 234 together with a fragmentation peak due to the loss of Me_2CO, CH_3CHO, Me and Cl residues (see Materials and methods section).

Stereostructure of chlorosphaeropsidone

These data indicate that compound **1** had a structure of 6-chloro-4,5-dihydroxy-3-methoxycyclohex-2-en-1-one.

The structure and the relative stereochemistry of **1** was confirmed by X-ray analysis, as shown in

Fig. 4. The molecular fragment consisted of the atom sequence O(1)-C(1)-C(2)-C(3)-C(4) and the methoxy group O(2)-C(7) was planar within 0.04 A. The six-membered ring exhibited a half-chair conformation. The absolute stereochemistry of chlorosphaeropsidone (**1**) was deduced by circular dichroism measurements. Its CD spectrum showed a clear positive Cotton effect at 248 nm which was also observed with sphaeropsidone (Evidente *et al*., 1998) and other related 5-hydroxy-7-oxabicyclo[4.1.0] hept-3-en-2-ones (Nagasawa *et al*., 1978), indicating a β-configuration for the 4-hydroxy group. This finding, together with the NMR and X-ray data, made it possible to assign the absolute configuration as depicted in **1**, which was designated as 6(*S*)-chloro-4(*S*),5(*R*)-dihydroxy-3-methoxycyclohex-2-en-1-one.

Chemical characterization of epichlorosphaeropsidone

As deduced from the spectroscopic data (EI-MS and NMR), epichlorosphaeropsidone (**2**) had the same molecular formula of $C_7H_9ClO_4$, as chlorosphaeropsidone, and gave IR, UV, ^1H and ^{13}C NMR and EI-MS readings very similar to those of **1**, indicating that **1** and **2** were isomers.

Compounds **1** and **2** showed different optical rotations and chromatographic behaviours in three systems: namely TLC on silica gel (eluents chloroform:*iso*-propanol, 9:1 and petroleum ether-acetone 1:1) and TLC on reverse phase (eluent ethyl alcohol:H_2O, 45:55). Therefore, it was possible to hypothesize that **2** was a diastereomer of **1**. Comparison of the ^1H NMR spectra of **1** and **2** showed the downfield shift ($\Delta\delta$ 0.30) of H-5 at δ 4.44 and the coupling between H-5 and H-6 ($J_{5,6}$=8.2 Hz in **1** and $J_{5,6}$=2.9 Hz in **2**) as the only differences. These data suggested that **2** was the C-6 epimer of **1**, and this is in agreement with the CD spectra. Chlorosphaeropsidone **2** showed the expected positive Cotton effect at 260 nm and different behaviour at a higher wavelength without any definite effect.

This structure was further confirmed by converting chlorosphaeropsidone (**1**) into **2** by enoli-

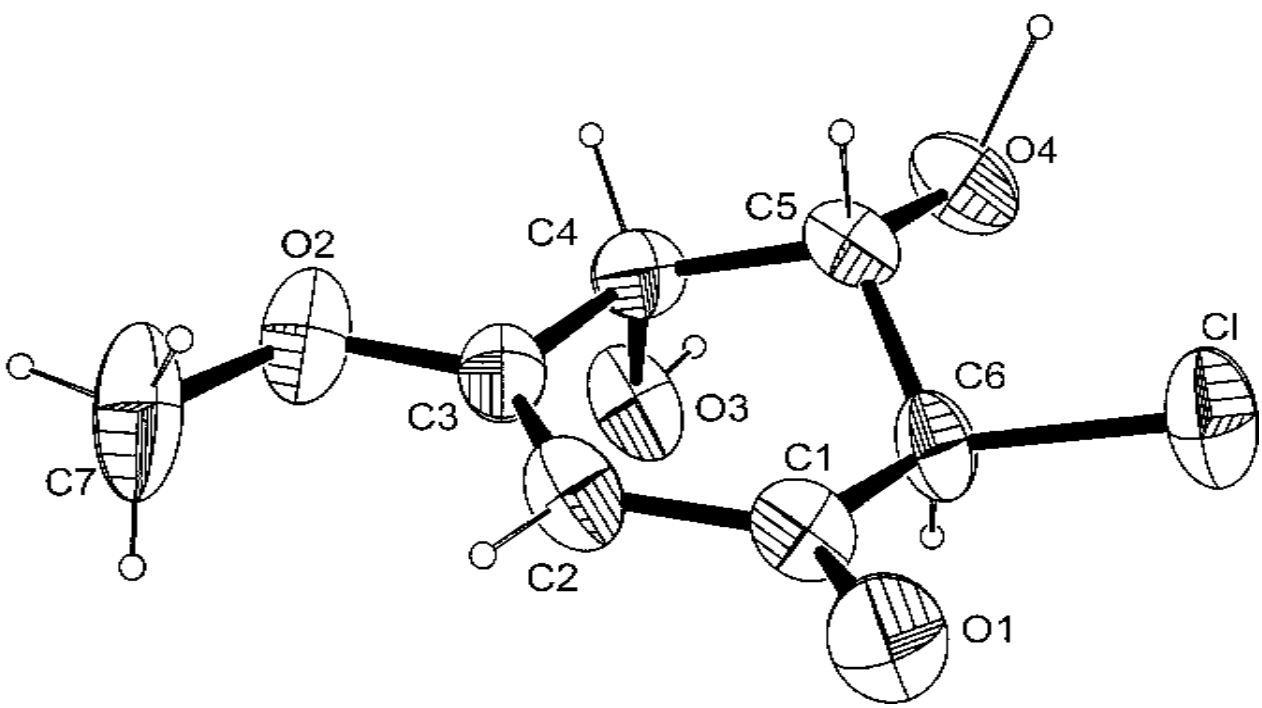

Fig. 4. Perspective view of chlorosphaeropsidone (**1**). Relevant bond lengths (A) and angles (°) are as follows : C(1)-C(2) 1.430(6), C(2)-C(3) 1.340(6), C(3)-C(4) 1.506(5), C(4)-C(5) 1.504(6), C(5)-C(6) 1.515(6), C(6)-C(1) 1.514(5), C(2)-C(1)-C(6) 116.(4), C(1)-C(2)-C(3) 121.9(3), C(2)-C(3)-C(4) 123.2(4), C(3)-C(4)-C(5) 110.7(3), C(4)-C(5)-C(6) 110.1(3), C(5)-C(6)-C(1) 110.8(4).

zation in aqueous sodium bicarbonate. The reaction was carried out on **1** in the conditions stated above to convert 6(*S*)-chloro-4(*R*),5(*R*)-dihydro-2-hydroxymethylcyclohex-2-en-1-one into the corresponding 6-epimer. These are two metabolites structurally related to **1** and **2** and isolated from culture filtrate of *Phyllostica* sp. (Sakamura *et al*., 1975). In this reaction, **1** in part remained unchanged and in part converted into **2** and into sphaeropsidone (**5**); the probable mechanism of these two convertions is shown in Fig. 5. The formation of sphaeropsidone, whose stereochemistry has recently been established (Evidente *et al*., 1998), confirmed the stereostructure of **1** as well as that of **2**, which can be designated as 6(*R*)-chloro-4(*S*),5(*R*)-dihydroxy-3-methoxycyclohex-2-en-1-one. The interchange between **1** and **2** was also observed when both metabolites were TLC-analysed and may be due to the acidity of the silica gel and to H_2O traces present in the solvent used.

Furthermore, a comparison of the ^1H NMR data of **1** and **2** with those obtained under the same experimental conditions for *trans*-bromohydrin (**4**), which will be described elsewhere, showed very different values for the constant of coupling between H-5 and H-6. The derivative **4** was prepared from sphaeropsidone by nucleophilic substitution with lithium tetrabromonickelate (II). This is a source of "soft" nucleophilic bromide which reacts regioselectively with epoxides to give bromohydrins in high yields (Dawe *et al*., 1984). In *trans*-bromohydrin the bromo and the hydroxy residues, attached to the C-6 and the C-5 respectively, assumed an axial-orientation and therefore the value of 5.3 Hz, typical for an equatorial-equatorial coupling, was observed between H-5 and H-6 (Sternhell, 1969). In **1** and **2** the same coupling yielded the values of 8.2 and 2.9 Hz, typical for

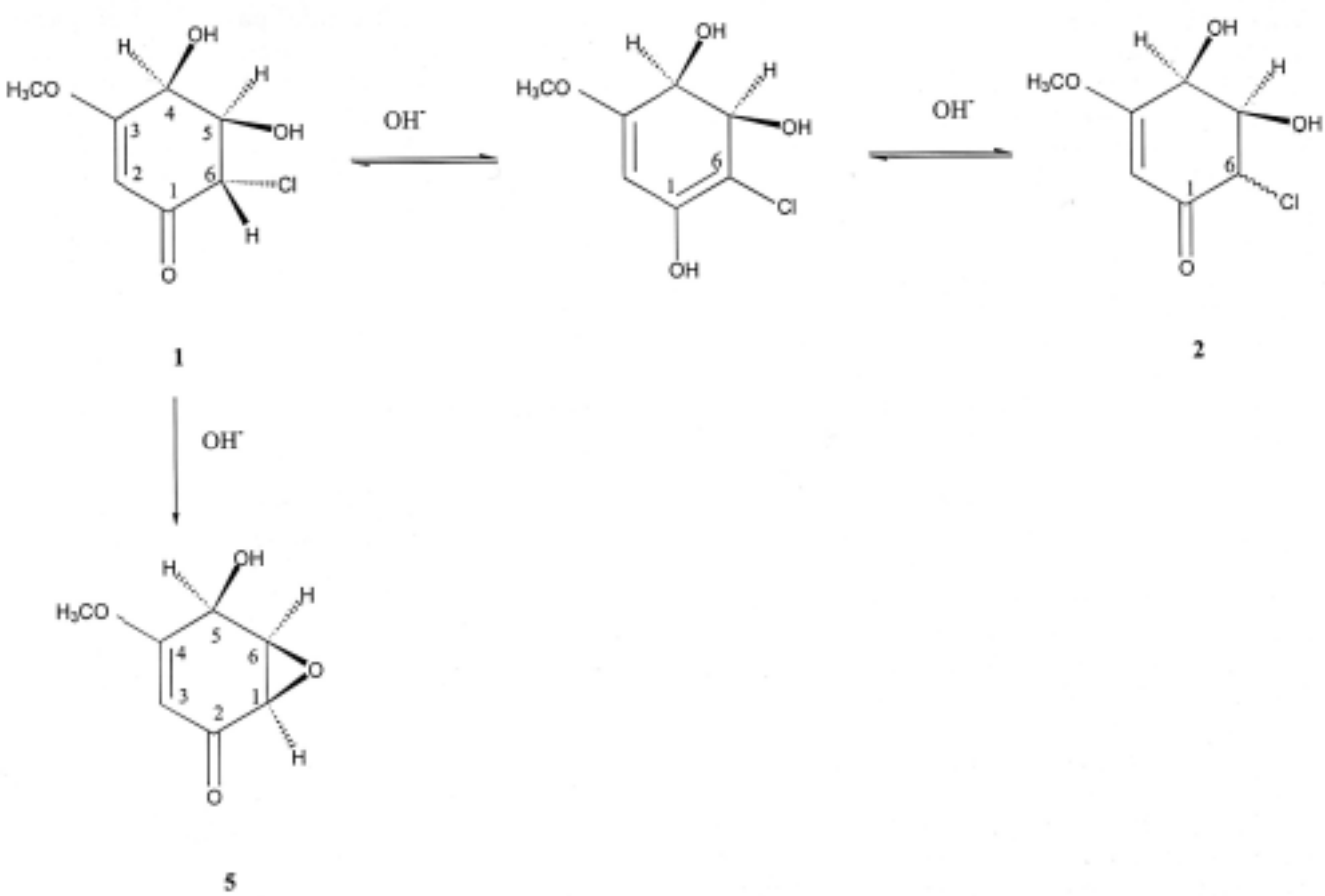

Fig. 5. Alkaline conversion of chlorosphaeropsidone (**1**) into epichlorosphaeropsidone (**2**) and sphaeropsidone (**5**).

an axial-axial and axial-equatorial orientation respectively (Sternhell, 1969). The relative stereochemistry thus deduced for **1** was in full agreement with that obtained by X-ray analysis as shown in Fig. 4. Moreover, the values of 3.7 and 3.6 Hz observed for the coupling between H-4 and H-5 in **1**, **2** and **4**, indicated that the latter proton assumed an axial-orientation in **1** and **2** and an equatorial-orientation in bromohydrin (**4**). Consequently the geminal hydroxy group at C-4 always had a β-configuration and this was also in agreement with the Cotton effect observed in the CD spectra of **1** and **2**.

Conclusions

The generation of artifacted molecules could easily occur during the extraction, purification and even chemical characterization of metabolites of microbial origin. It has, for example, been observed in the case of some 6-choloro-4,5-dihydroxycyclohex-2-en-1-ones closely related to the compounds that are the subject of this paper (Sakamura *et al*., 1975). The suspicion that **1** and **2** are artefacts generated by sphaeropsidone could therefore not be excluded. However, the data we odtained strongly suggested that **1** and/or **2** were fungal metabolites probably generated from sphaeropsidone by an enzymatic opening of the epoxy ring. In that case the other diastereomeric dimedone methyl ether would also be formed by alkaline enolization as depicted in Fig. 5. The natural origin of **1** and **2** was also supported by the fact that the fungal culture filtrate lacked chlorosphaeropsidones which could have been generated from episphaeropsidone (**6**).

The dimedone methyl ether nature of these two new fungal metabolites (**1** and **2**) as well as their absolute stereochemistry thus appear to have been demonstrated. They contain a carbon skeleton also found in other closely related fungal metabolites (Miller, 1968; Sakamura *et al.*, 1975; Nagasawa *et al.*, 1978; Fex and Wickberg, 1981; Turner and Aldridge, 1983; Evidente *et al.*, 1998). The lack of phytotoxicity by the two metabolites is probably due to the opening of the epoxy ring. It is known that the epoxy ring is a structural feature important for the biological activity of those fungal metabolites that contain it such as some cytochalasins and trichothecens (Cole and Cox, 1981; Vurro *et al.*, 1997). Therefore, an investigation into the structure-activity relationships of the sphaeropsidins, sphaeropsidones and their derivatives, including the two chlorosphaeropsidones (**1** and **2**) and *trans*-bromohydrin (**4**) is a necessary step to support our observations. A possible effect of **1** and **2** in synergy with or in addition to phytotoxic sphaeropsidins and sphaeropsidones which are metabolites produced by the same fungal pathogen should also be investigated.

S. sapinea f. sp. *cupressi* appears to be a further example of a phytopathogenic fungus which produces a number of toxins belonging to different families of natural products. This has already been observed for *Seiridium* spp., which also infect cypress (Evidente and Motta, 2000).

Acknowledgements

This investigation was supported in part by the National Research Council of Italy (CNR) Special Projet "Diagnosi precoce di malattie in piante di interesse agrario e forestale" paper N. 2, in part by grants from the Italian Ministry of University and Scientific Technological Research. The authors thank the "Centro di Metodologie Chimico-Fisiche" dell'Università di Napoli Federico II for NMR and X-ray analyses and Dr. O. Taglialatela of the "Centro Interdipartimentale di Analisi Strumentale" dell'Università di Napoli Federico II for HR-FAB and EI-MS spectra. We are also grateful to Mr. L. Scarola and Mr. C. Iodice, for technical assistance. Contribution N.185 (DISCA).

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Accepted for publication: May 30, 2000