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SPHAEROPSIDONE AND EPISPHAEROPSIDONE, PHYTOTOXIC DIMEDONE METHYL ETHERS PRODUCED BY *SPHAEROPSIS SAPINEA* F. SP. *CUPRESSI* GROWN IN LIQUID CULTURE

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Key Word Index—*Cupressus sempervirens*; cypress; *Sphaeropsis sapinea* f.s. *cupressi*; cypress canker disease; phytotoxins; tetraketides; dimedone methyl ethers; sphaeropsidone; episphaeropsidone.

Abstract—Two phytotoxic dimedone methyl ethers, named sphaeropsidone and episphaeropsidone, were isolated from *Sphaeropsis sapinea* f. sp. *cupressi*, a phytopathogenic fungus causing a canker disease of Italian cypress (*Cupressus sempervirens* L.). The same fungus produced the sphaeropsidins A, B and C, which are three phytotoxins recently chemically characterized as pimarane diterpenes. Sphaeropsidone and episphaeropsidone were characterized, using essentially spectroscopic methods, as two new phytotoxic disubstituted 7-oxabicyclo[4.1.0]hept-3-en-2-ones, which are epimers at C-5. Assayed on severed twigs of cypress, sphaeropsidone caused browning and necrosis on *Cupressus macrocarpa*, no symptoms on *C. sempervirens* and chlorosis on *C. arizonica*. Episphaeropsidone caused necrosis on *C. macrocarpa*, browning and necrosis on *C. sempervirens* and necrosis on *C. arizonica*. On the non-host plant tomato, both phytotoxins caused wilting. In a microbial assay, both compounds showed an inhibitory effect on the growth of five fungal species tested. The growth of *Verticillium dahliae* was enhanced by both dimedone methyl ethers. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Sphaeropsis sapinea f.sp. *cupressi* is a fungal pathogen which causes stem and branch canker of Italian cypress (*Cupressus sempervirens* L.) in areas limited to the Mediterranean coast. Such a canker differs from *Seiridium* canker, which is a common disease of cypress in the Mediterranean area [1].

Recently, the main phytotoxin and two toxic structurally related metabolites were isolated from culture extracts of *S. sapinea* f. sp. *cupressi*. All of them were chemically characterized as pimarane diterpenes, and were named sphaeropsidins A, B and C, respectively. The latter was identified as a new tricyclic acid pimarane diterpene [1, 2]. Moreover, it was demonstrated for the first time that *Diplodia mutila*, another ubiquitous fungus which induces canker formation, produced sphaeoropsidins A and C [2].

The organic extracts of the culture filtrates of *S. sapinea* f. sp. *cupressi* contained lower concentrations of at least three other phytotoxic substances. Two of them appeared to be strictly related compounds which were more polar than sphaeropsidins, and from which they were structurally different.

This paper describes the isolation, and chemical and biological characterization of new toxic metabolites, which proved to be epimeric dimedone methyl ethers. They were called sphaeropsidone and episphaeropsidone (**1** and **2**, respectively). The structures and the relative configuration were confirmed by X-ray analysis while the absolute stereochemistry was deduced by circular dichroism and NMR.

RESULTS AND DISCUSSION

The crude oily residue (1.20 g) obtained by extraction of the culture filtrates (3 l) of *S. sapinea* f. sp. *cupressi* with EtOAc was fractionated using a combination of CC and prep TLC to yield sphaeropsidins A, B and C and two more polar metabolites (**1** and

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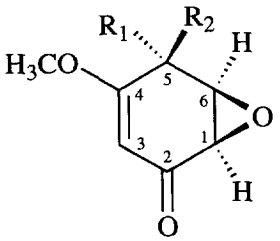

1 R_1=H, R_2=OH

2 R_1=OH, R_2=H

2), as shown by TLC (silica gel, eluent systems A and B). Preliminary spectroscopic investigations on metabolites **1** and **2**, obtained as a solid and an oil (15.7 and 23.3 mg l^{-1}), respectively, showed that they had a very similar structure which was completely different from that of the sphaeropsidins. Therefore, on this basis and on the basis of the spectroscopic data shown below, they were named sphaeropsidone and episphaeropsidone (**1** and **2**).

When assayed on test plants, sphaeropsidone and episphaeropsidone induced symptoms quite different from those previously described for sphaeropsidins. They appeared to be less toxic than all the sphaeropsidins. A 0.2 mg ml^{-1} solution of sphaeropsidone tested on severed cypress twigs caused browning and necrosis on *C. macrocarpa*, no symptoms on *C. sempervirens* and chlorosis on *C. arizonica*. A 0.2 mg ml^{-1} solution of episphaeropsidone absorbed by severed twigs of the same cypress species induced necrosis on *C. macrocarpa*, browning and necrosis on *C. sempervirens* and necrosis on *C. arizonica*. These results show that the three cypress species have a different level of sensitivity to the toxin. Moreover, sphaeropsidone appeared less phytotoxic than episphaeropsidone. The non-host test plant tomato was more sensitive to both compounds. In fact, the cuttings which had absorbed a 0.1 mg ml^{-1} solution of each dimedone methyl ether showed wilting symptoms three days after toxin treatment.

Sensitivity to sphaeropsidone and episphaeropsidone varied with the fungal species tested (Table 1). The most sensitive to sphaeropsidone were *Seiridium cupressi* and *Phomopsis amygdali*, the least sensitive *Seiridium cardinale*, *S. unicorne* and *Botrytis cinerea*. Episphaeropsidone affected the growth of the same fungal species more than sphaeropsidone. The mycelial growth of *P. amygdali*, *S. cupressi* and *B. cinerea* was inhibited more than that of *S. cardinale* and *S. unicorne*. By contrast, the mycelial growth of *Verticillium dahliae* was enhanced by both compounds. In all cases, sphaeropsidone appeared to be less active than episphaeropsidone. In addition, the inhibitory effect elicited by sphaeropsidins on two cypress pathogens *S. cardinale* and *S. cupressi* was shown by episphaeropsidone only on the latter fungus.

Sphaeropsidone (**1**) was assigned the molecular formula $C_7H_8O_4$ (HR-EIMS). Its IR spectrum showed bands characteristic of hydroxy groups and those very typical of a carbonyl and an olefinic group of a vinylogous ester [3]. The latter structural feature was consistent with the absorption maximum observed in the UV spectrum at 257 nm, which is characteristic of β-methoxy-α,β-unsaturated cyclohexenone [4]. The ^1H NMR spectrum (Table 2) showed the presence of a doublet at δ 5.23, due to an olefinic proton (H-3), which coupled with a proton (H-1) appearing at δ 3.48. The latter belongs to a *cis*-disubstituted oxiran ring and in the COSY-45 spectrum [5] coupled with the proton (H-6) at δ 3.84, resonating as a double doublet, due to further coupling with H-5, the proton adjacent to the secondary hydroxy group at δ 4.67 [6]. This appeared as a broad singlet being also coupled with the geminal hydroxy group present at δ 2.89 as a broad doublet [6]. In addition, the singlet of a methoxy group was observed at δ 3.76 [6]. These partial structures were confirmed by the ^{13}C NMR spectrum (Table 2) which showed the signal characteristic of the β-methoxy-α,β-unsaturated cyclohexenone skeleton at δ 192.8, 170.3 and 98.1 for the carbonyl and the quaternary methoxylated and the protonated carbons of the trisubstituted double bond, C-2, C-4 and C-3, respectively, while the methoxy group appeared at δ 56.5 [7]. The three oxygenated secondary carbons of the alcoholic and oxiran groups, which were assigned by the HMQC spectrum [8], appeared at the typical chemical shift values of δ 65.8 (C-5) and 53.7 and 53.1 (C-1 and C-6), respectively [7].

Considering these findings and the total of four unsaturations the structure of a 5-hydroxy-4-methoxy-7-oxabicyclo[4.1.0]hept-3-en-2-one was assigned to sphaeropsidone (**1**).

The structure was supported by the examination of its HR-EI mass spectrum which showed the $[M]^+$ at *m/z* 156.044262 ($C_7H_8O_4$) and peaks generated from fragmentation mechanisms typical of unsaturated cyclic ketones and methyl ethers [6]. In fact, the molecular ion by successive losses of Me and CO residues produced the ions at *m/z* 141 and 113.023968 ($C_5H_5O_3$). The molecular ion, by the alternative loss of HCO, yielded the ion at *m/z* 127.040234 ($C_6H_7O_3$). The FAB-MS showed the expected $[M+H]^+$ at *m/z* 157.

The structure and the relative stereochemistry of sphaeropsidone were confirmed by X-ray analysis, while its absolute stereochemistry was deduced by circular dichroism measurements. The CD spectra showed at 311 nm a negative Cotton effect as already observed in other structurally close compounds containing the 5-hydroxy-7-oxabicyclo[4.1.0]hept-3-en-2-one such as terremutin and panepoxydon [9, 10]. By contrast, chaloxone, epoxydon, (+)-epiepoxydon and (+)-desoxyepiepoxydon [10, 11] showed a positive Cotton effect in the same spectroscopic range. For all the compounds, that are fungal metabolites, the

Table 1. Sensitivity to sphaeropsidone and episphaeropsidone (**1** and **2**) of six phytopathogenic fungi grown on PSA medium at 25°, in the dark*

	Concentration of sphaeropsidones (**1**) and episphaeropsidones (**2**) ($\mu g\ ml^{-1}$)							
	[25]		[50]		[100]		L.S.D. ($p = 0.05$)	
Test fungus	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**
Botrytis cinerea	−13 (21.13)	−32 (34.45)	−16 (23.58)	−32 (34.45)	−10 (18.44)	−36 (36.87)	5.3	6.7
Phomopsis amygdali	−14 (21.97)	−18 (25.84)	−26 (30.66)	−23 (28.66)	−21 (27.28)	−42 (40.40)	6.8	8.1
Seiridium cardinale	0 (0.0)	0 (0.0)	−6 (14.18)	−6 (14.18)	−6 (14.18)	−6 (14.18)	4.5	5.7
Seiridium cupressi	−25 (30.00)	−31 (33.83)	−31 (33.83)	−33 (35.06)	−32 (34.45)	−34 (35.67)	5.2	5.8
Seiridium unicorne	0 (0.0)	−5 (12.92)	−5 (12.92)	−10 (18.44)	−10 (18.44)	−15 (22.79)	5.4	6.1
Verticillium dahliae	+25 (30.00)	+18 (25.10)	+25 (30.00)	+22 (27.97)	+40 (39.23)	+33 (35.06)	7.5	7.8

* Percentage of linear growth inhibition (−) or enhancement (+) was calculated by measuring the diameter of fungal colonies 1–2 weeks after inoculation. Experiments were repeated twice with three plates per species per toxin concentration. The figures are the means of six replicates. Angular transformations of percentage data are shown in parentheses. L.S.D. = Last Significative Difference.

Table 2. ^1H and ^{13}C NMR data of sphaeropsidone (**1**) and episphaeropsidone (**2**). The chemical shifts are in δ-values (ppm) from TMS

	1*		**2***	
C	δ_C	δ_H	δ_C	δ_H
1	53.7 *d*†	3.48 *dd*	51.8 *d*	3.42 *ddd*
2	192.8 *s*	—	193.6 *s*	—
3	98.1 *d*	5.23 *d*	98.7 *d*	5.23 *d*
4	170.3 *s*	—	172.4 *s*	— —
5	65.8 *d*	4.67 *br s*	65.3 *d*	4.64 *br d*
6	53.1 *d*	3.84 *dd*	53.7 *d*	3.72 *dd*
OMe	56.5 *q*	3.76 *s*	56.1 *q*	3.73 *s*
OH	—	2.89 *br d*	—	3.47 *d*

J (Hz) **1**: 1,3 = 1.9; 1,6 = 4.1; 5,6 = 3.3; 5,OH = 4.9; **2**: 1,3 = 1.4; 1,5 = 0.8; 1,6 = 3.7; 5,6 = 1.5; 5,OH = 6.2.

* 2D $^1H,^1H$ (COSY) and 2D $^{13}C,^1H$ (HMQC) NMR experiments delineated the correlations of all protons and the corresponding carbons.

† Multiplicities were determined by DEPT spectra.

absolute configuration has been reported [9–11]. The clear positive Cotton effect observed at 260 nm in the CD curve of **1**, indicated the spatial *syn*-relation of the hydroxy group at C-5 and the oxiran ring as already observed in some of the above mentioned metabolites [11]. In view of these findings the sphaeropsidone should have the absolute configuration as depicted in **1**.

Episphaeropsidone (**2**) had the same molecular formula, $C_7H_8O_4$, as sphaeropsidone as deduced from its HR-EIMS. The IR and UV absorptions were very similar to those of **1**. Furthermore, all the signal systems of both the ^1H and ^{13}C NMR spectra (Table 2), which were assigned fully by COSY and HMQC [5, 8], indicated that **2** had the same structural features as sphaeropsidone.

As expected, the HR-EIMS of **2** showed the $[M]^+$ at *m/z* 156.044502 ($C_7H_8O_4$), and, with respect to **1**, analogous fragmentation mechanisms. Therefore, the molecular ion generated ions at *m/z* 141 and 113.024826 ($C_5H_5O_3$) by successive losses of Me and CO, while by the alternative loss of HCO it produced the ion at *m/z* 127.040196 ($C_6H_7O_3$) [6]. As a confirmation its FAB-MS showed the $[M+H]^+$ at *m/z* 157.

Furthermore, episphaeropsidone, as compared to **1**, showed a different optical rotation and a different chromatographic behaviour in three different systems, namely normal (eluents B and C) and reverse phase (eluent D) chromatography. Therefore, it was possible to hypothesize that **2** was a diastereomer of **1**. In fact, an accurate comparison of the coupling constants between the systems of **1** and **2** showed as a substantial difference only the coupling between H-5 and H-6 ($J_{5,6}$ = 3.3 and 1.5 Hz in **1** and **2**, respectively) while those between the protons of the oxiran ring and the olefinic proton and H-1 remained unchanged. This difference suggested that **2** was the epimer at C-5 of **1**. This was supported by the close relation between

the coupling constants measured in epoxydon and panepoxydon [10], which have a *syn* and an *anti*-stereochemistry between the epoxy ring and the hydroxy group at C-5 as in **1** and **2**, respectively, and those here reported for the sphaeropsidone and epi-sphaeropsidone. It is noteworthy that only for **2** was a long-range coupling measured between H-1 and H-5 ($J_{1,5} = 0.8$ Hz) with a value very similar to that observed in panepoxydon for the analogous coupling [10].

The CD spectra of **2**, compared to **1**, showed the expected negative Cotton effect at 312 nm and a different behaviour at 257 and 235 nm confirming, in respect to **1**, the same orientation of the oxiran ring and that the hydroxy group at C-5 had an opposite configuration as depicted in **2**. Such a conclusion was also consistent with the results of a series of a ^{1}H NOE-difference spectra [12] performed on both **1** and **2**. As expected, in both metabolites a clear effect was observed between H-1 and H-6 and H-5 and the geminal OH group but only in **1** was there a significant effect between H-5 and H-6.

The dimedone methyl ether nature of the two structurally close sphaeropsidone and episphaeropsidone (**1** and **2**) appeared satisfactorily demonstrated. They contain a carbon skeleton found in other close related fungal metabolites [9–11, 13] some of which also exhibit phytotoxic activity [11].

EXPERIMENTAL

General

M.p.s: uncorr.; optical rotations: MeOH; CD: EtOH; IR and UV: KBr and neat and EtOH, respectively; ^{1}H and ^{13}C NMR: 500, 400 or 250 and, 125, 100 or 62.5 MHz, respectively, in $CDCl_3$, using the same solvent as int. standard. Carbon multiplicities were determined by DEPT spectra [7]. DEPT, COSY-45, HMQC, and NOE NMR experiments were performed using the Bruker software. EI and HR-EIMS: 70 eV; FABMS: glycerol/thioglycerol using Cs as bombarding atoms. Analytical and prep. TLC: silica gel (Merck, Kieslgel, 60 F_{254}, 0.25 and 0.50 mm, respectively) or on reverse phase: Whatman, KC18 F_{254}, 0.20 mm plates; spots were visualized by exposure to UV radiation and/or by spraying first with 10% H_2SO_4 in MeOH and then with 5% phosphomolybdic acid in MeOH, followed by heating at 110° for 10 min; CC: silica gel (Merck, Kieselgel, 60, 0.063–0.20 mm); solvent systems: (A) $CHCl_3$–*iso*–PrOH (19:1); (B) $CHCl_3$–*iso*–PrOH (9:1); (C) petrol–Me_2CO (1.5:1); (D) EtOH–H_2O (1.5:1). The petrol used had bp 40–70°.

Source of fungus and culture conditions

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

Production, extraction and purification of sphaeropsidones

The culture filtrates (3 l) of *S. sapinea* f. sp. *cupressi*, obtained as previously described [1], were acidified to pH 4 with 2 M HCl and extracted with EtOAc (4 × 1.5 l). The combined organic extracts were dried (Na_2SO_4) and evapd under red. pres. to give a red-brown oil residue (1.20 g) having a high phytotoxic activity. TLC analysis (silica gel, eluent system A) of this latter showed the presence of sphaeropsidins A, B and C [1, 2] at R_f 0.65, 0.43 and 0.50, respectively, and that of another main, more polar, metabolite at R_f 0.38. The crude residue was chromatographed by CC eluted with solvent system A to afford 9 groups of homogeneous frs, of which the groups 2–7 showed phytotoxic activity. As previously described, the combined residues (239.5 and 122 mg, respectively) left from fr. groups 3 and 4 and 6 and 7 yielded by fractionation in the already described conditions, sphaeropsidins A and B (240 and 80 mg), respectively. The residue (326 mg) of the fr. group 5 from the initial column, contained a residual amount of sphaeropsidin A, sphaeropsidin C and the other more polar metabolite (R_f 0.38). The latter by TLC on silica gel, but using the solvent system **B**, proved to be two metabolites with a very similar chromatographic behaviour (R_f 0.51 and 0.47, respectively). Therefore, the fractionation of these complex mixtures by CC column eluted with the same solvent system A yielded six groups of homogeneous fractions. The residues of the fr. groups 2–4 (52.2, 130, and 100 mg, respectively) were further purified by more steps of combined column and prep TLC (on silica gel) using both solvent systems A and B. This process gave further amounts of sphaeropsidin A (134 mg), sphaeropsidin C (40 mg) and the other two more polar metabolites, named sphaeropsidone (**1**, 47 mg, 15.7 mg l^{-1}) and epi-sphaeropsidone (**2**, 70 mg, 23.3 mg l^{-1}), respectively, as an amorphous solid and a homogeneous oil (R_f 0.47, 0.23 and 0.80 and R_f 0.51, 0.35 and 0.77, by silica gel and reverse phase TLC, eluent systems B, C and D, respectively). Sphaeropsidone crystallized from $CHCl_3$–MeOH as white needles, while epi-sphaeropsidone could not be crystallized.

Sphaeropsidone (**1**). White needles: m.p. 297–300°; $[\alpha]_d^{25}$ −130 (*c* 0.7); CD (*c* 7.1 10^{-5} M) $[\phi]^{25}$ (nm): −26,442 (311), +33,144 (260); UV λ_{max} nm (log ε): 257 (4.08); IR (KBr) ν_{max} cm^{-1}: 3387 1655, 1609; ^{1}H and ^{13}C NMR: Table 2. HR-EIMS *m/z* (rel. int.): 156.044262 ($C_7H_8O_4$, Calcd 156.042259, 100) $[M]^+$, 141 $[M-Me]^+$ (7), 127.040234 ($C_6H_7O_3$, Calcd

127.039519, 92) $[M-HCO]^+$, 113.023968 ($C_5H_5O_3$, Calcd 113.023869, 64) $[M-Me-CO]^+$, 111 (59), 95 (47), 85 (28). FABMS (+) *m/z* (rel. int.): 157 $[MH]^+$ (100).

Episphaeropsidone (**2**). Homogeneous oil $[\alpha]_d^{25}$ −240 (*c* 0.9); CD (*c* 4.8×10^{-5} M) $[\phi]^{25}$ (nm): −21,960 (312), +11,412 (257), +4.404 (235); UV λ_{max} nm (log ε: 254 (4.09); IR (neat) ν_{max} cm^{-1}: 3382, 1656, 1615; ^1H and ^{13}C NMR: Table 2. HR-EIMS *m/z* (rel. int.): 156.044502 ($C_7H_8O_4$, Calcd 156.042259, 100) $[M]^+$, 141 $[M-Me]^+$ (7), 127.040196 ($C_6H_7O_3$, Calcd 127.039519, 92) $[M-HCO]^+$, 113.024826 ($C_5H_5O_3$, Calcd 113.023869, 79) $[M-Me-CO]^+$, 111 (73), 95 (64). FABMS (+) *m/z* (rel. int.): 157 $[M+H]^+$ (100).

Toxin bioassays. Pure substances were assayed for phytotoxicity using severed twigs of *Cupressus sempervirens* var. *pyramidalis*, *C. macrocarpa* var. *lambertiana* and *C. arizonica*. For the experiments, the apical parts of the twigs, approximately 12 cm long, were used. The cuttings were taken from young cypress seedlings (3 year old) grown in the greenhouse at 25–27° and 60–70% R.H. During the bioassay, the severed twigs were maintained in a growth chamber at relatively low values of RH (60%), temp. (23°) and light (150 μmol m^{-2} s^{-1}). The phytotoxicity of both sphaeropsidones was also tested on a non-host herbaceous plant (tomato: *Lycopersicon esculentum* L. var. Marmande). Seedlings of tomato were grown in a growth chamber at 25° and 70–80% R.H. exposed to a luminous flux of 400 μmol m^{-2} s^{-1} with a 12 h photoperiod. Cuttings were taken of 21-day-old seedlings. The pure substances were tested at concns of 0.1–0.2 and 0.05–0.1 mg ml^{-1} on cypress and tomato cuttings, respectively. The toxicity of these solns was evaluated by placing the test plant parts (excised cypress twigs and tomato cuttings for 96 and 48 h, respectively) in the assay soln (3 ml) and then transferring them to distilled water. Symptoms developed within 2 and 21 days on tomato and cypress respectively.

Antifungal activity. The antimicrobial activity of both sphaeropsidones was tested on *Seiridium cardinale*, *S. cupressi* and *S. unicorne*, three causal agents of canker diseases on cypress, and on three phytopathogenic fungi: *Botrytis cinerea*, *Phomopsis amygdali* and *Verticillium dahliae*. A dilution series was prepared in the range 25–100 μg ml^{-1} toxic substance. The test was made by growing each fungal species in Petri dishes containing 10 ml of potato–dextrose (2%)-agar (PDA) supplemented with 25, 50 and 100 μg ml^{-1} toxic substance. The plates (3 per species) were seeded with 2 small pieces of a 10-day-old colony mat and incubated at 25° for 1–2 weeks, depending on the fungal species. The antifungal activity was evaluated by calculating the percentage of linear growth inhibition as previously reported [1] (Table 1).

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