PERGAMON

Phytochemistry 59 (2002) 817–823

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Sphaeropsidins D and E, two other pimarane diterpenes, produced in vitro by the plant pathogenic fungus *Sphaeropsis sapinea* f. sp. *cupressi*

Antonio Evidente[a,*], Lorenzo Sparapano[b], Giovanni Bruno[b], Andrea Motta[c]

[a]*Dipartimento di Scienze Chimico-Agrarie, Università di Napoli Federico II, Via Università 100, 80055 Portici, Italy*
[b]*Dipartimento di Biologia e Patologia Vegetale, Università di Bari, Via G. Amendola 165A, 70126 Bari, Italy*
[c]*Istituto di Chimica Biomolecolare del CNR, Comprensorio Olivetti, Edificio 70, Via Campi Flegrei 34, 80078 Pozzuoli, Italy*

Received 18 July 2001; received in revised form 20 December 2001

Dedicated to Professor Antonio Graniti on the occasion of his 75th birthday

Abstract

Two pimarane diterpenes structurally related to sphaeropsidins were isolated from the liquid culture of *Sphaeropsis sapinea* f. sp. *cupressi*, a plant pathogenic fungus causing a form of canker disease of Italian cypress (*Cupressus sempervirens* L.). The two metabolites, characterised by spectroscopic methods, were named sphaeropsidins D (0.40 mg l^{-1}) and E (0.16 mg l^{-1}). The same fungus produced sphaeropsidins A, B and C, sphaeropsidone and episphaeropsidone, which proved to be phytotoxic to cypress, and chlorosphaeropsidone and epichlorosphaeropsidone showing no phytotoxicity. Sphaeropsidin D assayed at 0.1 mg ml^{-1} on severed cypress twigs caused leaf browning and necrosis on *Cupressus macrocarpa*, but no symptoms were observed on *C. sempervirens* and *C. arizonica*. Symptoms appeared in a period of time (6 days after toxin-treatment) shorter than that for sphaeropsidin A. Sphaeropsidin E assayed at 0.2 mg ml^{-1} did not produce any symptom on the same cypress species tested with sphaeropsidin D.

Keywords: Cypress; *Cupressus sempervirens*; *Sphaeropsis sapinea* f. sp. *cupressi*; Cypress canker disease; Diterpenes; Pimaranes; Sphaeropsidins D and E

1. Introduction

Sphaeropsis sapinea f. sp. *cupressi*, the causal agent of a form of canker disease of the Italian cypress (*Cupressus sempervirens* L.) in Israel (Solel et al., 1987; Madar et al., 1989) and in Italy (Frisullo et al., 1997), produces in vitro several phytotoxic metabolites: the pimarane diterpenes sphaeropsidins A, B and C, the dimedone methyl ethers sphaeropsidone, episphaeropsidone, chlorosphaeropsidone and epichlorosphaeropsidone. Sphaeropsidins and sphaeropsidones are toxic to cypress, while chlorosphaeropsidone and epichlorosphaeropsidone lack phytotoxicity. The three sphaeropsidins show antimicrobial activity against some plant pathogenic fungi, particularly against *Seiridium cardinale* and *S. cupressi*, two micro-organisms that also cause canker to Italian cypress (Evidente et al., 1996, 1997). Sphaeropsidone and episphaeropsidone showed an antifungal activity lower than that of sphaeropsidins (Evidente et al., 1998).

This paper describes the isolation, chemical and biological characterisation of two new pimarane diterpenes, referred to as sphaeropsidins D (**4**) and E (**5**) that are structurally related to sphaeropsidins A (**1**), B (**2**) and C (**3**) (Evidente et al., 1996, 1997). Their structure was essentially determined by extensive use of NMR, MS and CD techniques, and by comparing these spectroscopic data with those of the known sphaeropsidins (Evidente et al., 1996, 1997).

2. Results and discussion

The culture filtrates of both strains of *S. sapinea* f. sp. *cupressi* were exhaustively extracted with EtOAc as previously described (Evidente et al., 2000). The crude

* Corresponding author. Tel.: +39-081-7885224; fax: +39-081-7755130.
E-mail address: evidente@unina.it (A. Evidente).

0031-9422/02/$ - see front matter
PII: S0031-9422(02)00015-8

Nomenclature

Sphaeropsidin D (**4**): 9α,11α-dihydroxy-7-oxopimara-8(14),15-dien-20-oic acid 6,20-lactone
Sphaeropsidin E (**5**): 7α,11β,14α-trihydroxy pimara-8(9),15-diene

organic extracts were combined and purified using CC and preparative TLC obtaining mainly sphaeropsidins A (**1**), B (**2**) and C (**3**) (Evidente et al., 1996, 1997), sphaeropsidones (Evidente et al., 1998) and chlorosphaeropsidones (Evidente et al., 2000). In particular, fraction 7 of the initial column yielded a residue (87.1 mg) from which we purified sphaeropsidones and a very low amount of two other metabolites. A TLC (silica gel, eluent system A) indicates that a compound (**4**) has the R_f 0.41 between sphaeropsidins and sphaeropsidones, while the other (**5**) with R_f 0.32 is between the latter and chlorosphaeropsidones. Preliminary, spectroscopic investigation on **4** (0.40 mg l^{-1}) and **5** (0.16 mg l^{-1}), obtained as homogeneous amorphous solids, indicated that they are closely related to the sphaeropsidins. Therefore, they were named sphaeropsiolins D and E.

Sphaeropsidin D (**4**) had a molecular weight of 362 corresponding to the molecular formula $C_{20}H_{26}O_6$, and proved to be structurally close to sphaeropsidins A (**1**). In fact, its ^1H and ^{13}C NMR spectra (Table 1) showed the decahydrophenanthrenone diterpene patterns, as already observed for **1** (Evidente et al., 1996). In particular, those of the hemiketal lactone between C-6 and C-10, the α,β-unsaturated ketone between C-7 and C-14, the tertiary hydroxylated carbon C-9, the vinyl group at C-13, and the tertiary methyl groups at C-4 and C-13. These structural features were consistent with the UV absorption maximum at 233 nm, characteristic of an α,β-unsaturated carbonyl group (Scott, 1964), and with the IR bands typical of this group as well as those of hydroxy and ester groups (Nakanishi and Solomon, 1977). The molecular formula of **4** differed from that of **1** for the presence of another oxygen, probably in a hydroxy group. In fact, accurate comparison of the ^1H NMR spectrum of **4** with that of **1** showed, as a substantial difference, the presence of a double doublet ($J = 11.9$ and 4.2 Hz) resonating at δ 4.32, a chemical shift typical of the hydrogen of hydroxylated secondary carbon of a cyclohexanol (Pretsch et al., 1989). In the COSY spectrum it appeared to be the X (H-11) part of the ABX system, whose AB system (CH_2-12) resonated as two double doublets ($J = 12.3$ and 11.9 and 12.3 and 4.2 Hz, respectively) centred at δ 1.88 and 1.67 (Sternhell, 1969). These results were consistent with the signals observed in the ^{13}C NMR spectrum (Table 1), which differed from that of **1** (Evidente et al., 1996) for the presence of a secondary hydroxylated carbon (C-11), appearing as a doublet at the typical chemical shift value of δ 66.2 and for the significant downfield shift ($\Delta\delta$ 22.9) of the adjacent methylene group (H_2C-12) observed as a triplet at δ 40.8 (Breitmaier and Voelter, 1987). Moreover, the absence of a signal in the aliphatic methylene group region was observed.

The above partial structure was localised between C-11 and C-12 by long-range COSY and TOCSY couplings observed between the olefinic proton H-14 (at δ 6.82) and the two H_2C-12 double doublets, as already observed in **1**, and by several ^1H,^{13}C correlations observed in the corresponding HMBC spectrum (Table 1). The coupling between the three quaternary carbons C-9, C-10 and C-13 at δ 73.0, 59.4 and 40.3, respectively, with H-11 and the two protons of H_2C-12 appeared to be particularly significant.

From COSY, TOCSY, HMQC and HMBC data, we assigned the chemical shifts to all protons and the corresponding carbons (Table 1), and suggested for sphaeropsidin D the structure of a 11-hydroxylated sphaeropsidin A (**4**). The structure assigned to **4** was also confirmed by the effects observed in the NOESY spectrum (Table 2).

The structure was also supported by HR–EI MS data, which showed the molecular ion at m/z 362.1762 and significant fragmentation peaks at m/z 344, 300, 285 and 267. They are generated from the parent ion by successive losses of H_2O, CO_2, Me and H_2O molecules. The same spectrum showed the peaks at m/z 318 and 282, which are alternatively produced from the molecular ion by consecutive losses of CO_2 and two molecules of H_2O (Pretsch et al., 1989).

Comparison of NMR and CD data of **4** with those of **1** (Evidente et al., 1996) showed that the two sphaeropsidins have the same absolute stereochemistry at C-5, C-6, C-9, C-10 and C-13, in agreement with the NOEs reported in Table 2 and depicted in their molecular formula. The coupling constants of H-11 with both H-12 and H-12′ allowed to locate this proton axial, and its geminal hydroxy group equatorial. The stereochemistry assigned to C-11 was in perfect agreement with the NOE correlations (Table 2) observed between H-11 and H-12′ and Me-17, and with the inspection of a Dreiding model of **4**.

Sphaeropsidin E (**5**) had a molecular weight of 320 corresponding to the molecular formula $C_{20}H_{32}O_3$. Preliminary spectroscopic investigation indicate for it some structural features already observed in sphaeropsidin C (**3**). Indeed, inspection of its ^1H and ^{13}C NMR spectra (Table 1) showed, as already observed for **3** (Evidente et al., 1997), the absence of the hemiketal lactone system and the extra presence of an aliphatic methylene and a methyl groups. They appeared in the

Table 1
^1H and ^{13}C NMR data of sphaeropsidins D and E (**4** and **5**, respectively). The chemical shift are in δ values (ppm) from TMS[a]

C[b]	**4**				**5**			
	δC[b]	δH	*J* (Hz)	HMBC	δC[b]	δH	*J* (Hz)	HMBC
1	26.1 *t*	2.33 *br d* 1.68 *m*	13.6	1.58	36.2 *t*	2.14 *br d* 1.65 *m*	11.3	1.20
2	18.7 *t*	1.58 *m* (2H)		2.33, 1.58	18.8 *t*	1.50 *m*, 1.20 *m*		
3	40.7 *t*	1.36 *br d* 1.17 *m*	12.7	2.33, 1.58, 1.20	41.6 *t*	1.47 *m*, 1.25 *m*		2.14, 0.93, 0.90
4	32.8 *s*			2.78, 1.58, 1.36,1.20	31.2 *s*			1.25, 0.93, 0.90
5	51.3 *d*	2.78 *s*		2.33, 1.20	46.2 *d*	1.25 *m*		1.84, 1.20, 0.93, 0.90
6	110.0 *s*			2.78, 1.20	28.6 *t*	1.84 *m* (2H)		1.25
7	192.8 *s*			6.82, 2.78	65.5 *d*	4.42 *br s*		4.03, 1.84
8	132.4 *s*			3.38	133.3 *s*			4.44, 4.03, 1.84
9	73.0 *s*			6.82, 2.78, 1.88, 1.67	146.4 *s*			4.42, 4.03, 1.91, 1.75, 1.20
10	59.4 *s*			4.32, 2.78, 2.33, 1.68, 1.58	59.3 *s*			1.47, 1.25
11	66.2 *d*	4.32 *dd*	11.9, 4.2	1.88, 1.67	65.5 *d*	4.44 *dd*	6.1, 5.6	1.91, 1.75
12	40.8 *t*	1.88 *dd* 1.67 *dd*	12.3, 11.9 12.3, 4.2		40.7 *t*	1.91 *dd* 1.75 *dd*	13.8, 6.1 13.8, 5.6	
13	40.3 *s*			6.82, 5.78, 5.10, 5.09, 4.32, 1.88, 1.67, 1.14	38.6 *s*			5.73, 5.09, 5.05, 4.44, 4.03, 1.91, 1.75, 1.17
14	152.4 *d*	6.82 *br s*[c]		5.78, 5.10, 5.09, 1.88, 1.67	72.7 *d*	4.03 *s*		5.73, 1.91, 1.75, 1.17
15	143.7 *d*	5.78 *dd*	17.5, 10.6	6.82, 5.10, 5.09, 1.88, 1.14	144.9 *d*	5.73 *dd*	17.6, 10.8	5.09, 5.05, 4.03, 1.95, 1.15
16	114.2 *t*	5.10 *dd* 5.09 *dd*	17.5, 1.5 10.6, 1.5	5.19	113.3 *t*	5.09 *dd* 5.05 *dd*	17.6, 0.94 10.8, 0.94	
17	25.9 *q*	1.14 *s* (3H)		6.82, 5.78, 1.88, 1.67	21.9 *q*	1.17 *s* (3H)		5.73, 4.03, 1.91, 1.75
18[d]	33.1 *q*	1.20 *s* (3H)		2.78, 1.17	33.3 *q*	0.93 *s* (3H)		
19[d]	23.1 *q*	1.20 *s* (3H)		2.78, 1.17	20.3 *q*	0.90 *s* (3H)		
20	174.9 *s*			2.78, 2.33, 1.68	21.9 *q*	1.20 *s* (3H)		
HO-6		5.19 *br s*			HO	3.71 *br s*		
HO-9, HO-11		3.38 *br s* (2H)						

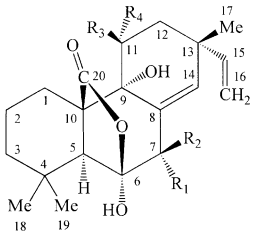
[a] 2D ^1H–^1H (COSY, TOCSY) and 2D ^{13}C, ^1H (HMQC) NMR experiments delineated the correlations of all protons and the corresponding carbons.
[b] Multiplicities determined by DEPT spectrum.
[c] This proton was long-range coupled with H-12 and H-12′ at δ 1.88 and 1.67, respectively.
[d] These assignments may be reversed.

Table 2
2D ^1H NOE (NOESY) data obtained for sphaeropsidins D and E (**4** and **5**)

4		**5**	
Considered	Effects	Considered	Effects
6.82 (H-14)	1.67 (H-12′), 1.14 (Me-17)	5.73 (H-15)	5.09 (H-16), 5.05 (H-16′), 4.03 (H-14), 1.91 (H-12), 1.17 (Me-17)
5.78 (H-15)	5.10 (H-16), 5.09 (H-16′), 1.67 (H-12′), 1.14 (Me-17)	5.09 (H-16)	5.73 (H-15), 1.91 (H-12), 1.17 (Me-17)
5.10–5.09 (H_2C-16)	3.38 (HO-9), 1.88 (H-12),	5.05 (H-16′)	5.73 (H-15), 1.91 (H-12), 1.17 (Me-17)
4.32 (H-11)	2.33 (H-1), 1.67 (H-12′), 1.14 (Me-17)	4.44 (H-11)	2.14 (H-1), 1.91 (H-12), 1.75 (H-12′), 1.65 (H-1′)
3.38 (HO-11)	1.88 (H-12), 1.67 (H-12′)	4.42 (H-7)	4.03 (H-14), 1.84 (H_2C-6), 1.20 (Me-20)
2.78 (H-5)	1.58 (H-2), 1.36 (H-3), 1.20 (Me-18 and/or Me-19)	4.03 (H-14)	5.73 (H-15), 4.42 (H-7), 1.91 (H-12), 1.17 (Me-17)
2.33 ((H-1)	1.67 (H-1′), 1.17 (H-3′)	2.14 (H-1)	1.65 (H-1′), 1.50 (H-2), 1.20 (H-2′ and/or Me-20)
		1.84 (H_2C-6)	4.42 (H-7), 1.25 (H-5), 0.93 (Me-18), 0.90 (Me-19)
		1.47 (H-3)	0.93 (Me-18), 0.90 (Me-19)

^{13}C NMR spectrum at the typical chemical shift value of δ 28.6 and 21.9, while the secondary carbon at the junction of the first two rings resonated, as expected, at δ 46.2 (Breitmaier and Voelter, 1987). The new methyl group (Me-20), which in the ^{13}C NMR spectrum overlapped with the Me-17 signal at δ 21.9 but resonated as a distinct singlet at δ 1.20 in ^{1}H NMR spectrum, was probably generated by the reductive opening of the lactone ring and should be linked to the quaternary carbon C-10. The H-5 singlet present in **1** was absent in the proton spectrum, but differing from **3**, the signal appeared shifted upfield ($\Delta\delta$ 1.18) and overlapping with the H-3′ signal at δ 1.25. Moreover, the protons of the adjacent methylene group (H_2C-6), generated from the reductive opening of the hemiketal lactone, resonated as complex multiplets shifted upfield ($\Delta\delta$ 0.85 and 0.62, respectively) at δ 1.84, respectively. These data together with COSY and TOCSY observations suggested a further coupling between the H_2C-6 protons and at least an adjacent proton at C-7, with the probable presence of an ABMX system. These results were consistent with the lack of any UV absorption maximum due to conjugated carbonyl groups and with the absence of IR bands typical of carbonyl groups. The IR spectrum only showed bands typical of hydroxy and olefinic groups (Nakanishi and Solomon, 1977). Moreover, when compared with sphaeropsidin C-5 showed more substantial differences. With respect to **3** its ^{1}H and ^{13}C NMR spectra showed the absence of the trisubstituted double bond signals, its conjugated carbonyl group and of the quaternary hydroxylated carbon, and the presence of signals from a tetrasubstituted double bond, and two further hydroxylated secondary carbons. The latter resonated as two singlets and two doublets at typical chemical shift values of δ 146.4 (C-9) and 133.3 (C-8) and δ 72.7 (C-14) and 65.5 (C-11) (Breitmaier and Voelter, 1987). The C-11 signal overlapped with that of C-7, in the spectrum recorded in $CDCl_3$. On the basis of these results another partial structure, two secondary hydroxylated carbons at C-7 and C-14 and a tetrasubstituted double bond between C-8 and C-9, was assumed. This hypothesis was consistent with the correlations observed in the COSY, TOCSY and HMQC spectra (Table 1). In fact, in the HMQC spectrum the hydroxylated secondary carbons at δ 72.7 (C-14) and 65.5 (C-7 and C-11) correlated with the singlet at δ 4.03 (H-14), the double doublet at δ 4.44 (H-11) and a broad singlet at δ 4.42 (H-7) (Pretsch et al., 1989). The latter is the X part of the above ABMX system as it correlated in the COSY and TOSCY spectra with the upfield shifted H_2C-6 and HC-5 complex multiplets at δ 1.84 and 1.25 (Sternhell, 1969; Pretsch et al., 1989). These, in turn, correlated in the HMQC spectrum with the corresponding carbons δ 28.6 (C-6) and 46.2 (C-5).

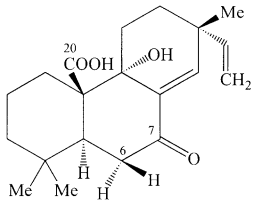
The double doublet ($J = 6.1$ and 5.5 Hz) observed at δ 4.44 (H-11) in the ^{1}H NMR spectrum was due to another hydroxylated secondary carbon (Pretsch et al., 1989), and belongs to another partial structure, already observed in **4** and that further differentiates **5** from **3**. In fact, this proton represents the X part of another ABX system, whose AB part (H_2C-12) resonated as two double doublets ($J = 13.8$ and 6.1 and 13.8 and 5.6 Hz, respectively) at δ 1.91 and 1.75 (Sternhell, 1969; Pretsch et al., 1989). These, in turn, in the HMQC spectrum correlated with the methylene carbon at δ 40.7 (Breitmaier and Voelter, 1987).



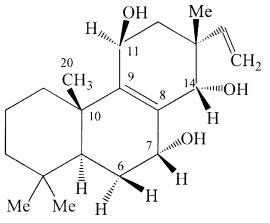
1 $R_1+R_2=O$, $R_3=R_4=H$

2 $R_1=R_3=R_4=H$, $R_2=OH$

4 $R_1+R_2=O$, $R_3=H$, $R_4=OH$



3



5

The last two partial structures were confirmed by one- and two-dimensional NMR spectra (^{1}H and ^{13}C NMR, COSY, HMQC and HMBC) of **5** recorded in C_6D_6. In a such solvent the H-7 and H-11 protons and the corresponding carbons were well resolved as a broad singlet and a triplet ($J = 6.1$ Hz) at δ 4.32 (H-7) and 4.16 (H-11), and at δ 65.3 (C-7) and 65.7 (C-11), respectively. Me-20 and Me-17 also had different ^{13}C NMR chemical shift values of 22.5 and 22.1 ppm, while the hydroxy groups appeared in the proton spectrum as a very sharp singlet at δ 6.13. However, in C_6D_6 of the aliphatic methylene and methyne sygnals in the proton spectrum became more complex preventing the assignment. It was obtained, both for protons and carbons, in $CDCl_3$ by using COSY, TOCSY, HMQC and HMBC spectra (Table 1).

These partial structures described were all consistent with the several ^{1}H,^{13}C long-range correlations observed in the HMBC spectrum (Table 1) and in the NOESY spectrum (Table 2).

On the basis of these results, the structure of a new tricyclic pimarane diterpene, 7α,11β,14α-trihydroxypimara-8(9)15-diene (**5**), was assigned to sphaeropsidin E.

This structure was also supported by HR EIMS data, which showed the molecular ion at m/z 320.2386 and significant fragmentation peaks at m/z 302, 284, 269 and 251. They were generated from the parent ion by consecutive losses of two H_2O, Me and a further H_2O residues. The other significant peak observed at m/z 287 was produced from the molecular ion by successive losses of H_2O and Me residues.

The stereochemistry of sphaeropsidin E was assigned as **4**. In fact, comparing the NMR and CD data of **5** with those of **3** and **2** (Evidente et al., 1997; Ellestad et al., 1972), in agreement with the NOE data reported in Table 2 we assigned the absolute stereochemistry at C-5, C-10 and C-13, which is reported in its structural formula. The coupling constants between H-7 and H_2C-6 and H-11 and H_2C-12 protons, respectively, allowed to locate both protons in the equatorial, and their geminal hydroxy group in the axial positions on B and C rings, which both adopt a pseudo-chair conformation. The stereochemistry assigned to C-7 and C-11 was in agreement with the observed NOEs (Table 2) (in particular, H-7 with Me-20, H_2C-6, and H-14 and H-11 with both protons of H_2C-1 and H_2C-12) and with the inspection of a Dreiding model of **5**. Similarly, H-14 was equatorial and its geminal hydroxy group axial, considering the significant NOEs observed between H-14 and H-7, H-15 and in particular Me-17, which is axial.

Sensitivity of various cypress species to sphaeropsidins D and E has proved different. Sphaeropsidin D, when assayed at concentrations ranging from 0.1 to 0.001 mg ml^{-1}, was toxic to *C. macrocarpa*. Symptoms appeared on the leaves 6 days after absorption of the toxic solution (0.1 mg ml^{-1}), and their severity increased during the following week. The leaves first showed chlorosis, turning yellow-brown and finally necrotic. The other two cypress species, *C. sempervirens* and *C. arizonica*, showed no sensitivity to sphaeropsidin D. When sphaeropsidin E was assayed at concentrations ranging from 0.2 to 0.02 mg ml^{-1} on twigs of the same cypress species, no phytotoxicity was recorded. Given the findings of Frisullo et al. (1997), which demonstrated that the artificial infections induced by the same strain of *S. sapinea* f. sp. *cupressi* caused cortical canker and dieback on seedlings of *C. macrocarpa*, *C. sempervirens* and *C. arizonica*, we may assume that the susceptibility of all three species tested did not correspond to their sensitivity to all the toxic metabolites produced by the pathogen.

The results obtained from the phytotoxicity assay indicated that toxicity of sphaeropsidins A, B, C and now D was associated with the presence of the double bond between C-8 and C-14 and probably also to that of the tertiary hydroxy group at C-9. Sphaeropsidin D, structurally related to sphaerosidin A, retained these features, while sphaeropsidin E, which differs from the other sphaeropsidins, did not. Moreover, spheropsidin E differed from the other sphaeropsidins also in the reduction to a methyl group (Me-20) of the carboxylic group at C-10.

In conclusion, the two sphaeropsidins D (**4**) and E (**5**) belong to unrearranged pimaranes. These are diterpenes already known as metabolites of plants, micro-organisms and marine organisms, some of which show interesting biological activity (McCrindle and Overton, 1969; Manitto, 1981; Turner and Aldridge, 1983; Hanson, 1985). Sphaeropsidin D proved to be structurally related to sphaeropsidin A, the main phytotoxin produced by the same plant pathogenic fungus (Evidente et al., 1996); sphaeropsidin E notably differs from the other sphaeropsidins (Evidente et al., 1996, 1997) and from the other known unrearranged pimaranes (McCrindle and Overton, 1969) for the functionalities of the phenanthrene ring system. This is the first report on sphaeropsidins D and E production by *S. sapinea* f. sp. *cupressi* in liquid culture, and on the biological activity of these two analogues of sphaeropsidins. The occurrence of sphaeropsidins D and E, independently from the phytotoxic action, may help to understand if changes in the molecular structure of sphaeropsidin A affects its biological activity on host and non-host plants, and its antifungal activity on plant pathogenic micro-organisms (Evidente et al., 1996, 1997). The understanding of the secondary metabolism of *S. sapinea* f. sp. *cupressi* could help to elucidate the taxonomic relationship between *S. sapinea* f. sp. *cupressi* and *S. sapinea*, questioned by Swart et al. (1993), and to support the pathogenicity data obtained by Frisullo et al. (1997) with the artificial infections induced by inoculating the same species. It is also important to point out that both strains of *S. sapinea* f. sp. *cupressi* of different origin (strain D3 from Morocco and strain 251.85/CBS from Israel) produced both sphaeropsidins D and E in addition to sphaeropsidins A, B and C and sphaeropsidones.

3. Experimental

3.1. General

The optical rotations were measured in MeOH solution on JASCO DIP-370 digital polarimeter, and the CD spectra were recorded in MeOH solution on a JASCO J-170 spectropolarimeter. IR and UV spectra were determined as neat and in MeOH solution, respectively, on a Perkin-Elmer IR FT-1720X spectrometer and a Perkin-Elmer Lambda 3B spectrophotometer. ^1H and ^{13}C NMR spectra were recorded at 500 and 100 MHz, respectively, in $CDCl_3$, on a Bruker spectrometer. The same solvent was used as internal standard. Carbon multiplicities were determined by DEPT spectra. DEPT, COSY-45, TOCSY, HMQC, HMBC and NOESY experiments were performed using

Bruker microprograms. EI MS and HR EIMS were taken at 70 eV on a Fisons Trio-2000 and a Fisons ProSpec spectrometer, respectively. Analytical and preparative TLC were performed on silica gel (Merck, Kieselgel 60 F_{254}, 0.25 and 0.50 mm, respectively) or reverse phase (Whatman, KC18 F_{254}, 0.20 mm) plates; the spots were visualised 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 at 110 °C for 10 min. CC: silica gel (Merck, Kieselgel 60, 0.063–0.20 mm). Solvent systems: (A) $CHCl_3$-*iso*-PrOH (19:1), (B) petrol-Me_2CO (2.3:1); (C) EtOH–H_2O (1:1); (D) *n*-hexane-*iso*-PrOH (5.6:1).

3.2. Fungal strain, culture medium and growth conditions

Two strains of *S. sapinea* f. sp. *cupressi* were used as inoculum. The first was isolated from the cortical tissues of naturally infected cypress (*Cupressus sempervirens*) trees collected in Morocco (Rabat; strain D3) and the second was purchased from Centraalbureau voor Schimmelcultures of Baarn (Netherland) strain 251.85/CBS *Diplodia pinea* f. sp. *cupressi* (Solel et al., 1987). Each single conidium isolate of *S. sapinea* f. sp. *cupressi* was grown on potato-sucrose-agar medium (PSA) on Petri dishes, and then transferred to slants containing the same substrate, 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. Each fungal strain was grown in stationary cultures in 1 l Roux flasks containing 150 ml of modified Czapek medium amended with 3% corn meal (pH 5.7). Each flask was seeded with 5 ml of a suspension of 14-day-old cultures in 50 ml sterile distilled water. The flasks were incubated at 25 °C for 30 days in darkness (Frisullo et al., 1997).

3.3. Production, extraction and purification of sphaeropsidins

The culture filtrates of both fungal strains (5 l each) of *S. sapinea* f. sp. *cupressi* were acidified to pH 4 with 2 M HCl and extracted with EtOAc (4×2.5 l). The two organic extracts were combined, dried (Na_2SO_4) and evaporated under reduced pressure to give a red-brown oily residue (2.84 g) having a high phytotoxic activity. Its TLC analysis on silica gel (eluent A) of this showed the presence of sphaeropsidins A, B and C (R_f 0.65, 0.43 and 0.50, respectively) (Evidente et al., 1996, 1997), sphaeropsidone and episphaeropsidone (R_f 0.38) (Evidente et al., 1998), the most polar chlorosphaeropsidones R_f 0.21 and 0.14 (Evidente et al., 2000), and that of other compounds with intermediate polarity between these groups of metabolites. The crude residue was chromatographed by CC, eluted with eluent A, to afford 9 groups of homogeneous fractions, as previously reported (Evidente et al., 2000). From purification of groups 2–6 residues by crystallization and combination of CC and preparative TLC (Evidente et al., 1997, 1998, 2000), pure sphaeropsidins A, B and C (681.9, 150 and 62.4 mg, respectively), sphaeropsidone and episphaeropsidone (26.4 and 12.2 mg, respectively) were obtained, as well as chlorosphaeropsidone and epichlorosphaeropsidone (44.0 and 19.7, respectively) from the residue (234 mg) of fraction 8. The residue (87.1 mg) of fraction 7, containing traces of sphaeropsidins, further amounts of sphaeropsidones, and other metabolites having an intermediate polarity between the latter and the sphaeropsidins and chlorosphaeropsidones (R_f 0.41 and 0.32, respectively), was purified by prep. TLC on silica gel (eluent B) giving further amounts of spheropsidones and episphaeropsidones (17.6 and 75 mg, respectively), and a homogeneous compound (R_f 0.30, 8.8 mg). The latter, which by TLC on reverse phase (eluent C) proved to be a mixture of two metabolites (R_f 0.54 and 0.15), was purified by prep. TLC in the same conditions giving sphaeropsidins D and E (**4** and **5**, 4.0 and 1.6 mg, 0.40 and 0.16 mg l^{-1}, respectively) as homogeneous amorphous solids. **4** and **5** showed an R_f of 0.41, 0.30, 0.59 and 0.54 and 0.32, 0.30, 0.52 and 0.15, respectively by TLC on silica gel, eluents A, B and D and on reverse phase, eluent C.

3.4. Sphaeropsidin D (*4*)

Compound **4** had: $[\alpha]_D^{25}$ +45.3° (*c* 0.06); CD $\Delta\varepsilon_{271}$ +1.8; $\Delta\varepsilon_{236}$ −2.7; $\Delta\varepsilon_{210}$ +2.3 (*c* 5.6 × 10^{-4} M), IR ν_{max} cm^{-1} 3508, 1729, 1716, 1638, 1214; UV λ_{max} nm (log ε): 233 (3.98); ^1H and ^{13}C NMR: Table 1; HR EIMS (rel. int) *m/z*: 362.1762 ($C_{20}H_{26}O_6$, calc. 362.1729, 56) $[M]^+$, 344 $[M–H_2O]^+$ (9), 318 $[M–CO_2]^+$ (19), 300 $[M–H_2O–CO_2]^+$ (9), 285 $[M–H_2O–CO_2–Me]^+$ (16), 282 $[M–CO_2–2xH_2O]^+$ (26), 267 $[M–2xH_2O–CO_2–Me]^+$ (28), 165 (87), 137 (100).

3.5. Sphaeropsidin E (*5*)

Compound **5** had: $[\alpha]_D^{25}$ +24.0° (*c* 0.12); CD $\Delta\varepsilon_{280}$ +1.6; $\Delta\varepsilon_{255}$ −1.7; $\Delta\varepsilon_{236}$ +1.5; $\Delta\varepsilon_{219}$−1.8; $\Delta\varepsilon_{209}$ +2.7 (*c* 1.3 × 10^{-3} M); IR ν_{max} cm^{-1} 3362, 1637; UV λ_{max} nm <220; ^1H and ^{13}C NMR: Table 1; HR EIMS (rel. int) *m/z*: 320.2386 ($C_{20}H_{32}O_3$, calc. 320.2351, 1) $[M]^+$, 302 $[M–H_2O]^+$ (25), 287 $[M–H_2O–Me]^+$ (13), 284 $[M–2xH_2O]^+$ (18), 269 $[M–2xH_2O–Me]^+$ (23), 251 $[M–3xH_2O–Me]^+$ (50), 234 (98), 219 (100).

3.6. Toxin bioassay

Culture filtrates, their chromatographic fractions and pure substances were assayed for phytotoxicity on three species of host plants (*Cupressus sempervirens* L. var.

pyramidalis, *C. macrocarpa* Hart. var. *lambertiana* and *C. arizonica* Gr.) as reported (Evidente et al., 1996). The purified sphaeropsidins D and E were tested at 0.001, 0.01 and 0.1 mg ml^{-1} and at 0.2–0.02 mg ml^{-1}, respectively. Controls included distilled water.

Acknowledgements

This investigation was supported in part by the National Research Council of Italy (CNR) Special Project "Diagnosi precoce di malattie in piante di interesse agrario e forestale" paper N.15, in part by grants from the Italian Ministry of Scientific and Technological Research (MURST). The authors thanks Mr. Vincenzo Mirra, (ICB-CNR, Pozzuoli) and L. Scarola (Dipartimento di Biologia e Patologia Vegetale, Università di Bari) for technical assistance and the "Servizio di Spettrometria di Massa del CNR e dell'Università di Napoli Federico II", for mass spectra; the assistance of the staff is gratefully acknowledged.

References

Breitmaier, E., Voelter, W., 1987. Carbon-13 NMR Spectroscopy. VCH, Weinheim.

Ellestad, G.A., Kunstmann, M.P., Mirando, P., Morton, G.O., 1972. Structure of fungal diterpene antibiotics LL-S491β and γ. Journal of the American Chemical Society 94, 6206–6208.

Evidente, A., Sparapano, L., Andolfi, A., Bruno, G., Giordano, F., Motta, A., 2000. Chlorosphaeropsidone and epichlorosphaeropsidone, two new chlorinated dimedone methyl ethers isolated from liquid cultures of *Sphaeropsis sapinea* f. sp. *cupressi*. Phytopathologia Mediterranea 39, 299–309.

Evidente, A., Sparapano, L., Fierro, O., Bruno, G., Giordano, F., Motta, A., 1997. Sphaeropsidins B and C, phytotoxic pimarane diterpenes from *Sphaeropsis sapinea* f. sp. *cupressi* and *Diplodia mutila*. Phytochemistry 45, 705–713.

Evidente, A., Sparapano, L., Fierro, O., Bruno, G., Giordano, F., Motta, A., 1998. Sphaeropsidone and episphaeropsidone, phytotoxic dimedone methyl ethers produced by *Sphaeropsis sapinea* f. sp. *cupressi* grown in liquid culture. Phytochemistry 48, 1139–1143.

Evidente, A., Sparapano, L., Motta, A., Giordano, F., Fierro, O., Frisullo, S., 1996. A phytotoxic pimarane diterpene of *Sphaeropsis sapinea* f. sp. *cupressi*, the pathogen of a canker disease of cypress. Phytochemistry 42, 1541–1546.

Frisullo, S., Bruno, G., Lops, F., Sparapano, L., 1997. Un nuovo agente di cancro del cipresso in Italia. Petria 7, 141–158.

Hanson, J.R., 1985. Terpenoids. In: Thomson, R.H. (Ed.), The Chemistry of Natural Products. Blackie, Glasgow, pp. 154–189.

Madar, Z., Solel, Z., Kimchi, M., 1989. Effect of water stress in cypress on the development of cankers caused by *Diplodia pinea* f. sp. *cupressi* and *Seiridium cardinale*. Plant Disease 73, 484–486.

Manitto, P., 1981. Biosynthesis of Natural Products. Hellis Horwood Limited, Chichester.

McCrindle, R., Overton, K.H., 1969. The diterpenoids, sesterterpenoids and triterpenoids. In: Coffey, S. (Ed.), Rodd's Chemistry of Carbon Compounds, Vol. II, Part C. Elsevier, Amsterdam, pp. 369–403.

Nakanishi, K., Solomon, P.H., 1977. Infrared Absorption Spectroscopy, 2nd Edition. Holden Day, Oakland.

Pretsch, P.D.E., Clerc, T., Seibl, J., Simon, W., 1989. Tables of Spectral Data for Structure Determination of Organic Compounds. Springer-Verlag, Berlin.

Scott, A.I., 1964. Interpretation of the Ultraviolet Spectra of Natural Products. Pergamon Press, Oxford.

Solel, Z., Madar, Z., Kimchi, M., Golan, Y., 1987. Diplodia canker of cypress. Canadian Journal Plant Pathology 9, 115–118.

Sternhell, S., 1969. Correlation of interproton spin-spin coupling constant with structure. Quarterly Review 23, 237–269.

Swart, W.J., Wingfield, M.J., Grant, W.S., 1993. Comparison of *Sphaeropsis sapinea* and *Sphaeropsis sapinea* f. sp. *cupressi*. Mycological Research 97, 1253–1260.

Turner, W.B., Aldridge, D.C., 1983. Fungal Metabolites II. Academic Press, London.