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A PHYTOTOXIC PIMARANE DITERPENE OF *SPHAEROPSIS SAPINEA* F. SP. *CUPRESSI*, THE PATHOGEN OF A CANKER DISEASE OF CYPRESS*

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Key Word Index—*Cupressus sempervirens*; *Cupressaceae*; cypress; *Sphaeropsis sapinea* f. sp. *cupressi*; cypress canker disease; phytotoxins; diterpenes; pimaranes; sphaeropsidin A.

Abstract—A phytotoxic metabolite, named sphaeropsidin A, is produced *in vitro* by *Sphaeropsis sapinea* f. sp. *cupressi*, a fungus that causes a canker disease of Italian cypress (*Cupressus sempervirens*). The spectroscopic data indicates that sphaeropsidin A is identical with a pimarane diterpenoid antibiotic previously isolated from some *Aspergillus* spp. When absorbed by severed twigs of three species of *Cupressus* and cuttings of two herbaceous plants, sphaeropsidin A at 0.1 mg ml^{-1} produced leaf yellowing, browning and dieback and yellowing of leaf, necrosis and epinasty, respectively. Subperidermal injection of a 0.1 mg ml^{-1} solution of sphaeropsidin A into young cypress trees caused longitudinal fissures and dark brown discolouration of cortical tissues. The compound showed antimicrobial activity towards 12 fungal species when assayed at a concentration range from 10 to 100 μg ml^{-1}. This is the first report showing that sphaeropsidin A is a non-selective fungal phytotoxin.

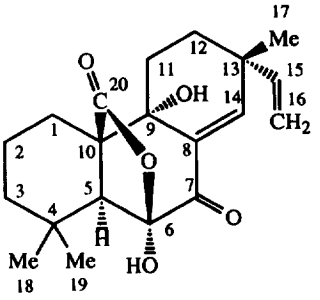
INTRODUCTION

We have reported previously on the isolation, and chemical and biological characterization of several phytotoxins produced by three species of *Seiridium* (*S. cardinale*, *S. cupressi* and *S. unicorne*) [1–3], pathogenic for cypresses. The fungi associated with canker diseases of Italian cypress (*Cupressus sempervirens* L.) and other species of *Cupressus* in the Mediterranean area belong to the genera *Diplodia*, *Pestalotiopsis*, *Seiridium* and *Sphaeropsis* [4–11].

Stem and branch canker of Italian cypress trees (*Cupressus sempervirens* L.) has been recently described by Solel and coworkers [7, 8], and the fungus isolated from infected tissues was referred to as *Sphaeropsis sapinea* f. sp. *cupressi*. The cankers produced by this fungus differ from *Seiridium* canker, i.e. a fissure develops and becomes a deep crack through the entire bark but not into the wood, allowing light brown drops of resin to flow. The taxonomy of *S. sapinea* (Fr.:Fr.) Dyko & Sutton, an opportunistic pathogen of *Pinus* species in more than 25 countries [10], has been the subject of considerable confusion. Most recently, the results obtained by Swart *et al.* [11] provided substantial evidence for the lack of a close relationship between *S. sapinea*, more closely related to *Diplodia mutila*, a pine pathogen, and *S. sapinea* f. sp. *cupressi*, a physiological form of *S. sapinea*. Usually species belonging to the order of *Sphaeropsidales* produce *in vivo* and *in vitro* toxic substances [12]. The finding that *S. sapinea* f. sp. *cupressi* is able to produce *in vitro* phytotoxic metabolites may help lead to an understanding of the role of these compounds in the expression of symptoms induced by the fungus on the infected cypress plants, and to distinguish *S. sapinea* f. sp. *cupressi* from *S. sapinea* on the basis of their metabolic behaviour. Furthermore, the study of the effects of such substances on other virulent pathogens of cypress such as *Seiridium* spp. during the infection process is also of interest.

This paper reports on the isolation of the main phytotoxin, sphaeropsidin A (**1**), purified from culture filtrates of *S. sapinea* f. sp. *cupressi*. The physical and spectroscopic data of **1** are very similar to those partially reported for the antibiotic pimarane diterpene isolated from *Aspergillus chevalieri* [13]. The structure and relative configuration of sphaeropsidin A were confirmed by X-ray analysis. In addition the phyto-

*Dedicated to Professor A. Ballio on the occasion of his 75th birthday.



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toxicity and the antifungal activity of this toxin on two phytopathogens of cypress and other phytopathogenic fungi are described for the first time.

RESULTS AND DISCUSSION

The culture filtrates of *S. sapinea* f. sp. *cupressi* were exhaustively extracted with EtOAc to give a red-brown oil having a high phytotoxic activity. The latter was fractioned by silica gel column chromatography, as described in Experimental, to yield the main metabolite, sphaeropsidin A (**1**).

When assayed on test plants sphaeropsidin A caused yellowing, browning and necrosis on *Cupressus sempervirens*, dieback on *C. macrocarpa* and browning and necrosis on *C. arizonica*. The herbaceous plants were also affected by the toxin: yellowing and necrosis appeared on *Avena sativa* cv. Park and *Lycopersicon esculentum* cv. Marmande. When injected into the cortical tissues of cypress plants mentioned above, sphaeropsidin A produced a longitudinal fissure, or crack, and a dark brown discolouration on the more sensitive species (*C. sempervirens* and *C. macrocarpa*) and a dark brown discolouration of the internal tissues of *C. arizonica* (Table 1).

The antimicrobial activity of sphaeropsidin A was assayed on the growth in culture of 12 species of fungi (Table 2) and conidial germination of two *Seiridium* species (Table 3). *Fusicoccum amygdali* proved to be more sensitive than any of the other fungi tested while *Colletotrichum acutatum*, *Fusarium solani*, *Pyrenochaeta lycopersici* and *Verticillium dahliae* appeared to be less sensitive to the toxin with a percentage linear growth inhibition between 25.8 and 28.7% (Table 2). The antimicrobial activity of sphaeropsidin A may help the saprophytic survival of these fungi in their natural substrates, or when they live as parasites within plant tissues.

The fungistatic activity of sphaeropsidin A against *S. cardinale* and *S. cupressi*, both causal agents of a canker disease of cypress as *S. sapinea* f. sp. *cupressi*, is sufficient to account for the antagonistic action of *S. sapinea* f. sp. *cupressi* against *Seiridium* species. Sphaeropsidin A affected the growth, sporulation, conidial germination and pigmentation of both fungi (Table 3). If sphaeropsidin A is produced *in vivo* by the fungus, it can be postulated that *S. sapinea* f. sp. *cupressi* spreading along the stem or branches may prevent the invasion of cortical tissues by *S. cupressi* or *S. cardinale*.

The molecular formula, $C_{20}H_{26}O_5$, determined for sphaeropsidin A (**1**) by HR-EI-mass spectrum analysis indicated the presence of eight unsaturations, four of which were attributable to the α,β-unsaturated carbonyl group, the vinyl and the lactone groups, as shown by the characteristic absorptions present in the IR spectrum together to those typical of hydroxy groups [14]. The presence of an α,β-unsaturated carbonyl group was confirmed by the absorption maximum observed in the UV spectrum at 247 nm [15]. These data also suggested the presence of four rings in **1**. The ^1H and ^{13}C NMR spectra (Table 4) confirmed these structural features [16, 17] while extensive 1D and 2D NMR experiments, using COSY-45, TOCSY (Correlated and Total Correlation Spectroscopy), HMQC experiments [18] performed in different solvents ($CDCl_3$, CD_3OD, $DMSOd_6$ and $DMSOd_6$–D_2O), allowed the assignment of all protons and the corresponding carbons (Table 4). By using HMBC, NOESY and ROESY [18], we established the structure of sphaeropsidin A as that of the diterpenoid depicted in formula **1**. This structure was supported by the fragmentation ions observed in the HR-EI-mass spectrum at *m/z* 328.1619 ($C_{20}H_{24}O_4$), 284 and 269 (base peak), which are generated from the molecular ion at *m/z* 346.1821 ($C_{20}H_{26}O_5$) by the consecutive losses of H_2O, CO_2

Table 1. Symptoms caused by sphaeropsidin A (**1**) on test plants

Test plants	Severed twigs*	Cuttings*	Bark stem
Host plants:			
Cupressus sempervirens	Yellowing, browning and necrosis		Longitudinal fissure and dark† brown discolouration
Cupressus macrocarpa	Dieback		Crack and dark brown discolouration
Cupressus arizonica	Browning and necrosis		Dark brown discolouration†
Non-host plants:			
Avena sativa cv. Park		Yellowing and necrosis	Brown discolouration
Lycopersicon esculentum		Epinasty and necrosis	Brown discolouration and stewing

*Symptoms on severed twigs of three cypress species or cuttings of herbaceous test plants after toxin absorption.
†Symptoms after injection of toxin into the cortical tissue of 3-year-old cypress plants.

Table 2. Sensitivity to sphaeropsidin A (**1**) of 12 phytopathogenic fungal species grown on PDA medium at 25°, in the dark*

Test fungus	Concentration of sphaeropsidin A ($\mu g\ ml^{-1}$)					L.S.D. ($P = 0.05$)
	10	25	50	75	100	
Seiridium cardinale	12.2	34.5	50.7	52.4	54.1	
	(20.36)	(35.97)	(45.40)	(46.38)	(47.35)	(10.2)
Seiridium cupressi	14.7	35.8	49.8	55.2	59.8	
	(22.55)	(36.75)	(44.89)	(47.98)	(50.65)	(8.7)
Botrytis cinerea	10.5	32.4	43.4	49.7	56.7	
	(18.91)	(34.70)	(41.21)	(44.83)	(48.85)	(9.2)
Colletotrichum acutatum	8.7	15.6	25.1	25.4	26.7	
	(17.16)	(23.26)	(30.07)	(30.26)	(31.11)	(11.2)
Fusarium oxysporum	7.6	12.8	15.8	22.6	28.2	
	(16.00)	(20.96)	(23.42)	(28.38)	(32.08)	(7.5)
Fusarium solani	8.2	16.5	19.9	23.5	27.9	
	(16.54)	(23.97)	(26.49)	(29.00)	(31.88)	(8.4)
Fusicoccum amygdali	23.7	52.8	70.0	73.2	77.0	
	(29.13)	(46.61)	(56.79)	(58.82)	(61.34)	(12.1)
Penicillium expansum	7.2	12.3	19.2	34.3	41.2	
	(15.56)	(20.53)	(25.99)	(35.85)	(39.93)	(7.5)
Pyrenochaeta lycopersici	10.5	18.4	20.7	21.4	25.8	
	(18.91)	(25.40)	(27.06)	(27.56)	(30.53)	(6.8)
Sclerotinia minor	28.9	54.3	60.3	61.5	64.6	
	(32.52)	(47.47)	(50.94)	(51.65)	(53.49)	(7.4)
Sclerotinia sclerotiorum	12.8	31.7	40.7	43.7	47.4	
	(20.96)	(34.27)	(39.64)	(41.38)	(43.51)	(6.7)
Verticillium dahliae	8.7	17.6	20.5	24.8	28.7	
	(17.16)	(24.80)	(26.92)	(29.87)	(32.39)	(6.5)

*Percentage of linear growth inhibition 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.

and Me [16, 19]. The structure **1** obtained from HR EIMS and NMR has also been confirmed by X-ray, and the data will be published elsewhere.

Sphaeropsidin A is an unrearranged pimarane, and thus belongs to a group of diterpenes already known as plant, microorganism and marine organism metabolites, some of which have interesting biological activity [20–23]. However, from all the above data **1** appears identical to the antibiotic labelled as LL-S491β previously isolated from *A. chevalieri*, *A. flaschentraegeri* and *A. cristatus* [13, 21]. In fact, sphaeropsidin A showed physical (mp, $[\alpha]^{25}D$ and CD) and spectroscopic (UV, IR and the very few reported ^1H NMR data) properties very similar to those of the antibiotic LL-S491β when these were measured in the same conditions, as reported in ref. 13.

EXPERIMENTAL

General. Mps: uncorr.; optical rotations: MeOH; IR and UV: KBr and MeOH, respectively; CD: MeOH; ^1H and ^{13}C NMR: $CDCl_3$, CD_3OD, $DMSOd_6$ or $DMSOd_6$–D_2O at 500 or 400 and 125 or 100 MHz, respectively, using the solvent as the int. standard.

Table 3. Effects of sphaeropsidin A (**1**) on biological characteristics of two species of *Seiridium*

Species	Toxin concentration ($mg\ ml^{-1}$)	Colony diameter* on PSA at 23° (mm)	Sporulation	Inhibition of conidial† germination (%)	Pigmentation
S. cardinale	0.1	28.5±4.2	None	2	Olive-greenish
	0.05	35.4±4.3	None	2	Greenish
	0.01	42.6±3.5	None	4	Pearl gray
	0	62.4±5.2	Moderate	4	Pearl gray
S. cupressi	0.01	24.7±3.1	Sparse	1	Orange
	0.05	30.4±3.1	Sparse	2	Orange
	0.01	38.3±5.4	Moderate	4	Orange
	0	43.5±5.2	Abundant	4	Salmon

*Colony diameter (±SD), sporulation and pigmentation were measured/observed at the end of a growth period of 21 days.
†The degree of inhibition of conidial germination was evaluated as follows: 0 = no germination; 1 = less than 10%; 2 = 11–40%; 3 = 41–80% and 4 = 81–100%, i.e. complete germination.

Table 4. ^{1}H and ^{13}C NMR data ($CDCl_3$) of sphaeropsidin A (**1**). The chemical shifts are in δ-values (ppm) from TMS

C*	δ	Hδ*	J(Hz)	HMBC
1	22.8 *t*†	2.22 *br d*	10.7	2.70
1′		1.58 *m*		
2	17.9 *t*	1.58 *m*		
2′		1.58 *m*		
3	26.7 *t*	1.86 *m*		
3′		1.82 *m*		
4	32.2 *s*			2.70, 1.17 (1.16)
5	51.2 *d*	2.70 *s*		5.20, 2.22, 1.35, 1.17 (1.16)
6	103.6 *s*			5.20
7	191.6 *s*			6.82, 5.20, 2.70
8	132.9 *s*			1.84
9	70.9 *s*			6.82, 2.70, 1.84, 1.64, 1.58
10	57.0 *s*			2.70, 2.22, 1.64, 1.58
11	40.3 *t*	1.35 *br d*	14.1	2.22, 1.58
11′		1.18 *m*		
12	29.3 *t*	1.84 *m*		6.82, 5.83, 1.06
12′		1.64 *m*		
13	39.3 *s*			6.82, 5.83, 5.06, 1.84, 1.64, 1.06
14	152.6 *d*	6.82 *br s*‡		5.83, 1.84, 1.64, 1.18
15	144.2 *d*	5.83 *dd*	17.6, 10.5	6.82, 5.06
16	113.3 *t*	5.06 *dd*	17.6, 1.5	
16′		5.06 *dd*	10.5, 1.5	
17	24.3 *q*	1.06 (3H) *s*§		6.82, 5.83
18‖	32.5 *q*	1.17 (3H) *s*		1.58
19‖	22.3 *q*	1.16 (3H) *s*		2.70
20	174.7 *s*			5.20, 2.70, 2.22, 1.58
HO-6		5.20 *br s*		
HO-9		2.18 *br s*		

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

†Multiplicities were determined by DEPT.

‡This proton was long-range coupled with H-12 and H-12′ at δ 1.84 and 1.64, respectively.

§NOE effects were observed between the signals of Me-17 and those of H-14 and H_2C-16 in the NOESY spectrum recorded in CD_3OD.

‖These attributions may be reversed.

Carbon multiplicities were determined by DEPT [17]. COSY, TOCSY, HMQC, HMBC, NOESY and ROESY experiments were performed using Bruker standard microprograms; HR-EIMS: 70 eV; Analyt. and prep. TLC: silica gel (Merck, Kieselgel 60 F_{254}, 0.25 and 0.50 mm, respectively); spots were visualized by exposure to UV radiation and/or by spraying first with 10% H_2SO_4 in MeOH, followed by 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) EtOAc–*n*-hexane (2.3:1); (C) petrol–Me_2CO (2.3:1).

Fungal cultures. Sphaeropsis sapinea f. sp. *cupressi* was isolated from cortical tissues of infected cypress (*Cupressus sempervirens* L.) trees collected in Morocco and in Italy. Single spore isolates of *S. sapinea* f. sp. *cupressi* were grown on potato-sucrose (2%)–agar slants at 25° for 10 days and then stored at 5° in the fungal collection of the Dipartimento di Patologia vegetale, Università di Bari, Italy (N. 1527 and 1530, respectively).

Toxin extraction and purification. The isolate N. 1527 was grown in stationary culture in 11 Roux flasks containing 150 ml Czapek medium with the addition of 2% corn meal (pH 5.7). Each flask was seeded with 5 ml of a suspension of three culture tubes (10-day-old cultures) in 50 ml sterile medium. The flasks were incubated at 25° for 30 days in the dark. At harvest, the mycelium mat was removed by filtration. The culture filtrates (5 l; pH 7.1–7.3) were extracted with EtOAc (4 × 2.5 l). The combined organic extracts were dried (Na_2SO_4) and evapd to give a red-brown oil residue (3.86 g) having a high phytotoxic activity. TLC analysis (silica gel, eluent systems A, B and C, respectively) of the extract showed the presence of main metabolites at R_f 0.65, 0.74 and 0.58, respectively. The crude residue was chromatographed by CC eluted with solvent system A, producing 7 groups of homogeneous frs. Only the residue left from frs 1–4 showed phytotoxic activity. The amorphous solids from fractions 2–4 were combined (2.82 g) and crystallized from EtOAc–*n*-hexane yielding sphaeropsidin A (**1**) (1.26 g) as white needles. The residue left from the mother liquors was purified by combination of CC and prep. TLC (silica gel, solvent system A) to give a further amount of **1** (0.86 g, for a total of 0.42 g l^{-1}) as an amorphous solid.

Sphaeropsidin A (**1**) had: mp 180–185°; $[\alpha]_D^{25}$ +109.6 (*c* 2.0); CD, $\Delta\varepsilon_{247}$: −3.2; $\Delta\varepsilon_{275}$: +2.3: IR ν_{max} cm $^{-1}$: 3437, 3287, 1745, 1726, 1630; UV λ_{max} nm (log ε): 247 (3.66); ^{1}H and ^{13}C NMR: Table 4; [lit. 13: mp: 180–185°; $[\alpha]_D^{25}$ +112.4 (MeOH); CD $\Delta\varepsilon_{241}$: −3.7; IR (KBr) ν_{max} cm $^{-1}$: 3500, 1755, 1710, 1620; UV (MeOH) λ_{max} nm (log ε): 241 (3.77); ^{1}H NMR ($CDCl_3$): δ 5.84 (1H, four lines, H-15), 5.65–5.02 (2H, eight lines, 2H-16), 5.25 and 1.90 (HO-6 and HO-9), 2.74 (*s*, H-5), 1.20 (6H, *s*, Me-18 and Me-19) and 1.09 (3H, *s*, Me-17)]; HR-EIMS, *m/z* (rel. int.): 346.1821 ($C_{20}H_{26}O_5$, calcd 346.1773, 55) $[M]^+$, 328.1619 ($C_{20}H_{24}O_4$, 20) $[M - H_2O]^+$, 310 $[M - 2 \times H_2O]^+$ (6), 300.1763 ($C_{19}H_{24}O_3$, 21); $[M - H_2O - CO]^+$, 284 $[M - H_2O - CO_2]^+$ (36), 282 $[M - 2 \times H_2O - CO]^+$ (64), 269 $[M - H_2O - CO_2 - Me]^+$ (100).

Phytotoxin bioassays. Culture filtrates, their organic extracts, chromatographic fractions and pure substances were assayed for phytotoxicity using severed twigs of cypresses (*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 (3–5-years-old) grown in the greenhouse at 25–27° and 60–70% RH. The phytotoxicity of sphaeropsidin A was also tested on herbaceous non-host plants (tomato: *Lycopersicon esculentum* var. Marmande; oat: *Avena sativa* cv. Park). Seedlings of tomato and oat were grown in a growth chamber at 25° and 70–80% RH exposed to a luminous flux of 400 μmol m^{-2} s^{-1} with a 12 hr photoperiod. Cuttings were taken from 21-day-old seedlings. During the assay, the severed twigs of cypress trees and the cuttings of tomato and oat plants were maintained in a growth chamber at relatively low values of RH (60%), temp. (23°) and light (150 μmol m^{-2} s^{-1}). Aliquots of the culture filtrate were assayed after 1 : 10 000 dilution with distilled water. The crude extracts from culture filtrates and residues of CC fractions were assayed at concns of 0.1, 0.2 and 0.5 mg ml^{-1}, and pure substance at 10–100 μg ml^{-1}. The toxicity of these solns was evaluated by placing the test plant parts (excised cypress twigs for 96 hr, tomato and oat cuttings for 48 hr) in the assay soln (3 ml) and then transferring them to distilled water. Symptoms developed within 2, 4 and 21 days on tomato, oat and cypress, respectively (Table 1). A soln of sphaeropsidin A (0.1 mg ml^{-1}) was also injected into the cortical tissue of 3-year-old cypress plants, at a distance of 30 cm from the apex (Table 1).

Antifungal activity. The assays were carried out with *S. cardinale* and *S. cupressi*, two causal agents of canker disease on cypress and other phytopathogenic fungi such as: *Botrytis cinerea*, *Colletotrichum acutatum*, *Fusarium oxysporum*, *Fusarium solani*, *Fusicoccum amygdali*, *Penicillium expansum*, *Pyrenochaeta lycopersici*, *Sclerotinia minor*, *Sclerotinia sclerotiorum*, *Verticillium dahliae*, as test microorganisms. A dilution series was prepared in the range 10–100 μg ml^{-1} toxic substance. A first test was carried out with a conidial suspension (10^5–10^6 conidia ml^{-1} depending on the test fungus) in the dilution series. The plastic trays containing 500 μl spore suspension per well were incubated at 25° for 48 hr. A further test was made by growing each fungal species in Petri-dishes containing 20 ml of potato-dextrose agar (PDA) supplemented with 10, 25, 50, 75 and 100 μg ml^{-1} toxic substance. The plates (5 per fungal 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 effect of toxin was evaluated by calculating the percentage of linear growth inhibition as 100 $(y - x)/y$ where y = mean colony diameter of toxin-free cultures and x = mean colony diameter of toxin-containing cultures (Table 2). In addition, the cultural characteristics of *S. cardinale* and *S. cupressi* grown in the presence of sphaeropsidin A were compared. The isolates were tested at least twice. Triplicate plates of each isolate on potato-sucrose agar (PSA) were incubated in the dark at 23° for 3 weeks. Colony diameters were measured to the nearest mm after dark incubation and colony characteristics visually scored in the following way: a) shape or symmetry of growth extension; b) colony texture; c) colour based solely on objective judgement of the variety and intensity of pigmentation; d) sporulation based on visual appraisal of conidiomata of conidia production coupled with microscopic examination for confirmation of conidia production. The percentage of conidial germination (assessed by an arbitrary 0–4 scale) was also calcd (Table 3).

Nomenclature. Sphaeropsidin A (**1**): 9H-10,4a-(Epoxymethano)phenanthrene-9,12-dione, 7-ethenyl-1,2,3,4,4b,5,6,7,10,10a-decahydro-4b,10-dihydroxy-1,1,7-trimethyl.

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