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SPHAEROPSIDINS B AND C, PHYTOTOXIC PIMARANE DITERPENES FROM *SPHAEROPSIS SAPINEA* F. SP. *CUPRESSI* AND *DIPLODIA MUTILA*

ANTONIO EVIDENTE,* LORENZO SPARAPANO,† OLGA FIERRO, GIOVANNI BRUNO,† FEDERICO GIORDANO,‡ and ANDREA MOTTA§

Dipartimento di Scienze Chimico-Agrarie, Universitá di Napoli Federico II, Via Universitá 100, 80055 Portici, Italy; †Dipartimento di Patologia Vegetale, Universitá di Bari, Via Amendola 165/A, 70126 Bari, Italy; ‡Dipartimento di Chimica, Universitá di Napoli Federico II, Via Mezzocannone 4, 80134 Napoli, Italy; §Istituto per la Chimica di Molecole di Interesse Biologico del CNR, Via Toiano 6, 80072 Arco Felice, Italy¶

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Key Word Index—*Cupressus sempervirens*; *Cupressaceae*; cypress; *Sphaeropsis sapinea* f.s. *cupressi*; *Diplodia mutila*; cypress canker disease; phytotoxins; diterpenes; pimaranes; sphaeropsidins B and C.

Abstract—Two phytotoxic pimarane diterpenes, named sphaeropsidins B and C, were isolated from two phytopathogenic fungi causing canker diseases of Italian cypress (*Cupressus sempervirens* L.): *Sphaeropsis sapinea* f. sp. *cupressi* and *Diplodia mutila*. *S. sapinea* f. sp. *cupressi* produced both phytotoxins, whereas *D. mutila* produced sphaeropsidins C and A, the latter also being reported as the main phytotoxin of *S. sapinea* f. s. *cupressi*. Characterized by chemical and spectroscopic methods, sphaeropsidin B proved to be a known fungal metabolite for the first time isolated from *S. sapinea* f. sp. *cupressi*, while sphaeropsidin C is a new tricyclic acid pimarane diterpene produced by both fungi. Assayed on severed twigs of cypress and oak, sphaeropsidins B and C caused dieback on *C. macrocarpa*, browning and necrosis on *C. sempervirens* and yellowing on *C. arizonica*, necrosis on *Quercus cerri* and *Q. ilex*, and browning and necrosis on *Q. robur*. When injected into cortical tissues of cypress and oak seedlings mentioned above, they caused on the former dark brown discolouration, browning of internal tissues and browning, and brown-blackish spots or necrotic lesions on the latter. On non-host plants like tomato and oat, sphaeropsidins B an C caused necrosis on cuttings and brown discolouration or stewing on the stem. In the antimicrobial assay, both compounds showed an inhibitory effect on mycelial growth of seven test fungi. © 1997 Elsevier Science Ltd

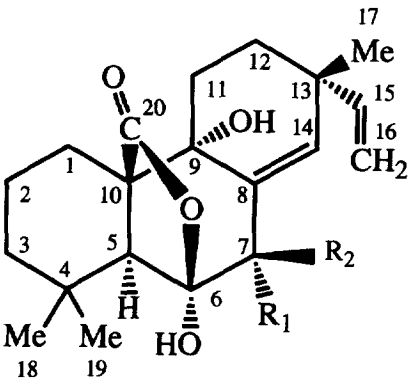
INTRODUCTION

Several phytopathogenic fungi belonging to the genus *Sphaeropsis* and *Diplodia* are responsible for severe disease of agrarian and forest trees. *S. sapinea* f. sp. *cupressi* and *D. mutila* are causal agents of canker diseases for Mediterranean cypress (*Cupressus sempervirens* L.), inducing symptoms very similar to those produced by three phytopathogenic *Seiridium* species, whose phytotoxins have been studied by our group in the last decade [1]. It is well known that phytotoxins may be involved in pathogenesis and that microbial toxins may be used in the biological control of other pathogens which infected the same host plant as the toxin-producer microorganism. It is possible that phytotoxins produced by *S. sapinea* f. sp. *cupressi* and *D. mutila* could be used as antimicrobial substances against *Seiridium* spp. Studies were carried out to isolate and characterize the toxins produced *in vitro* by these two fungi. The main toxin in culture filtrates of *S. sapinea* f. sp. *cupressi* was reported as sphaeropsidin A (**1**) [2], a pimarane diterpene. The antimicrobial and phytotoxic activities of this compound were also reported [2]. A new examination of the organic extract of the culture filtrates showed the presence of at least three minor metabolites. One of these was also present, together with sphaeropsidin A, in the culture extract of *D. mutila*.

This paper describes the isolation, the chemical and the biological characterization of two of these minor phytotoxic metabolites called sphaeropsidins B and C (**2** and **3**), both structurally related to sphaeropsidin A. Furthermore, sphaeropsidin A was identified for the first time as the main phytotoxin produced by *D. mutila*.

* Author to whom correspondence should be addressed.

¶ This institute is part of the 'Istituto Nazionale di Chimica dei Sistemi Biologici'.



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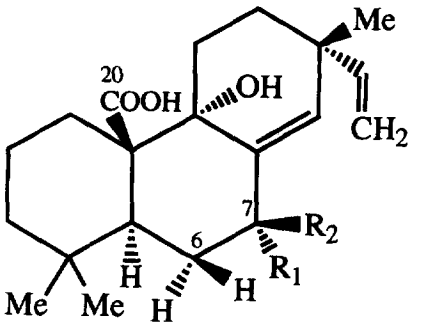

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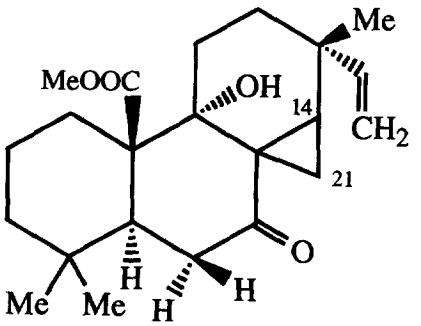

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RESULTS AND DISCUSSION

The crude oily residue (775 mg) obtained by extraction with ethyl acetate of culture filtrates of *S. sapinea* f. sp. *cupressi* was fractioned, using a combination of column chromatography and preparative TLC steps, to yield sphaeropsidin A (**1**, 14.0 mg l^{-1}) [2] and two (**2** and **3**, 8.0 and 2.5 mg l^{-1}, respectively) other metabolites more polar with respect to **1** when chromatographed by TLC (silica gel, eluent systems A and B). Only the chromatograms of the most polar (**2**) of those having an intermediate polarity was not UV-detectable, while the other (**3**) was revealable by UV-irradiation as **1**. Both metabolites **2** and **3** crystallized from a chloroform–methanol mixture. When the same procedure was applied on the crude organic extract (269 mg for 1.5 l of culture filtrates) of *D. mutila* only the main phytotoxin, identified as sphaeropsidin A, and the lesser polar metabolite (**3**), were isolated (27.0 and 9.3 mg l^{-1}, respectively). In a preliminary spectroscopic investigation the two metabolites with intermediate polarity showed a structure similar to that of **1** and therefore they were called sphaeropsidins B and C.

When assayed on test plants sphaeropsidins B and C induced symptoms (Table 1) similar to those observed assaying sphaeropsidin A. Both substances were shown to be non-selective toxins like sphaeropsidin A. They caused symptoms on host plants similar to those observed on naturally infected plants. A 0.1 mg ml^{-1} solution of sphaeropsidins B and C tested on severed cypress twigs caused dieback of *C. macrocarpa*, browning and necrosis on *C. sempervirens* and yellowing on *C. arizonica*. This finding shows that the three species have a different grade of tolerance to the toxin action. Injected into the cortical tissue of 3-year-old seedlings of the same cypress species, a 0.1 mg ml^{-1} solution of sphaeropsidins B and C caused a dark brown discolouration of *C. macrocarpa* tissues, browning of internal tissues or necrotic lesions of *C. sempervirens* and finally browning on *C. arizonica*. Oak tissues gave a different response to the toxins. On severed twigs of *Quercus cerri* and of *Q. ilex*, a 0.1 mg ml^{-1} solution of sphaeropsidins B an C caused necrosis whereas on *Q. robur* caused browning and necrosis. Cortical tissue of oak seedlings treated with 0.1 mg ml^{-1} solution of sphaeropsidin B or C, 3 weeks after absorption, showed symptoms of brown-black-

Table 1. Symptoms caused by sphaeropsidins B and C (**2** and **3**) on test plants

	Severed twigs*		Cuttings*		Stem†	
Test plant	**2**	**3**	**2**	**3**	**2**	**3**
Cypress host:						
Cupressus macrocarpa	Dieback	Dieback			Dark brown discolouration of cortical tissues	Dark brown discolouration of cortical tissues
Cupressus sempervirens	Browning and necrosis	Browning and necrosis			Browning of internal cortical tissues	Necrotic bark lesions
Cupressus arizonica	Yellowing	Yellowing			Browning	Browning
Oak host:						
Quercus cerri	Necrosis	Necrosis			Brown-blackish spots	Necrotic bark lesions
Quercus ilex	Necrosis	Necrosis			Bark cracks	Necrotic bark lesions
Quercus robur	Browning and necrosis	Browning			Brown-blackish spots	Brown-blackish spots
Non-host:						
Avena sativa			Necrosis	Necrosis	Brown discolouration	Blistering
Lycopersicon esculentum			Necrosis	Necrosis	Brown discolouration	Blistering

* Symptoms appeared on severed twigs of three cypress species or cuttings of herbaceous test plants after toxin (0.1 mg ml^{-1}) absorption

† Symptoms appeared after injection of toxins (0.1 mg ml^{-1}) into the cortical tissue of 3-year-old cypress plants.

Table 2. Sensitivity to sphaeropsidins B and C (**2** and **3**) of 7 phytopathogenic fungi grown on PSA medium at 25°C, in the dark*

	Concentration of sphaeropsidin B (**2**) and sphaeropsidin C (**3**) ($\mu g\ ml^{-1}$)										L.S.D. ($P = 0.05$)	
	10		25		50		75		100			
Test fungus	**2**	**3**	**2**	**3**	**2**	**3**	**2**	**3**	**2**	**3**	**2**	**3**
Botrytis cinerea	12.5 (20.7)	7.2 (15.6)	24.8 (29.9)	12.1 (20.3)	32.7 (34.9)	27.4 (31.6)	48.6 (44.2)	32.8 (34.9)	62.4 (52.2)	38.4 (38.3)	7.2	8.2
Colletotrichum acutatum	7.5 (15.9)	10.3 (18.7)	11.8 (20.1)	18.4 (25.4)	21.7 (27.8)	24.2 (29.5)	30.6 (33.6)	33.4 (35.3)	34.8 (36.1)	40.4 (39.5)	6.5	7.5
Fusarium oxysporum	8.4 (16.9)	8.1 (16.5)	13.6 (21.6)	11.7 (20.0)	18.7 (24.9)	15.3 (23.1)	22.7 (28.5)	21.2 (27.4)	30.2 (33.3)	27.8 (31.8)	8.7	7.2
Phomopsis (Fusicoccum) amygdali	20.2 (26.8)	11.4 (19.7)	31.1 (33.9)	18.2 (25.2)	38.4 (38.3)	26.6 (31.1)	44.6 (41.9)	34.8 (36.1)	63.5 (52.8)	41.7 (40.2)	10.5	8.8
Sclerotinia sclerotiorum	10.5 (19.9)	7.5 (15.9)	18.4 (25.4)	10.8 (19.2)	27.5 (31.6)	21.5 (27.6)	40.2 (39.3)	30.2 (33.3)	51.2 (45.7)	37.4 (37.7)	11.3	7.4
Seiridium cardinale	10.2 (18.6)	6.8 (15.1)	15.4 (23.1)	12.5 (20.7)	23.1 (28.7)	25.7 (30.5)	37.3 (37.6)	34.1 (35.7)	41.3 (39.9)	39.4 (38.9)	9.2	6.7
Seiridium cupressi	12.7 (20.9)	11.2 (19.5)	17.3 (24.6)	18.1 (25.2)	20.4 (26.8)	28.2 (32.1)	31.2 (33.9)	37.3 (37.6)	38.3 (38.2)	42.4 (40.6)	6.8	8.1

* 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. = least significative difference.

ish spots or necrotic bark lesions on *Q. cerri*, bark crack or necrotic lesions on *Q. ilex* and brown-blackish spots on *Q. robur*. The non-host test plants tomato and oat showed necrosis on leaves and brown discolouration or stewing on the stem.

Sensitivity to sphaeropsidins B and C varied with the fungal species tested (Table 2) The most sensitive to sphaeropsidin B were *Botrytis cinerea* and *Phomopsis (Fusicoccum) amygdali*, the less sensitive *Fusarium oxysporum*. Sphaeropsidin C appeared to be less active than sphaeropsidin B; in addition the inhibition effect on the mycelial growth was the same for all species. The inhibitory effects of both substances on two cypress pathogens *S. cardinale* and *S. cupressi* are promising for their potential use in the control of fungal pathogens of cypress to prevent early infections.

Sphaeropsidin B (**2**) showed a molecular weight of 348 as deduced from its EI and FAB mass spectra. The IR spectrum differed from that of **1** in the absence of the band due to a ketone group while in the UV spectrum it lacked the absorption maximum generated by the α,β-unsaturated ketone. These structural differences were confirmed by comparison of the ^1H NMR and ^{13}C NMR spectra of **2** (Tables 3 and 4, respectively) with those of sphaeropsidin A. The ^1H NMR spectrum showed as the only differences the presence of a doublet ($J_{7,14} = 2.4$ Hz) at δ 4.22, assigned to the proton (H-7) of a secondary hydroxylated carbon, and the upfield shift ($\Delta\delta$ 1.01) and the multiplicity of H-14, appearing as a double doublet ($J_{7,14} = J_{12,14} = 2.4$ Hz) at δ 5.81. In the ^{13}C NMR spectrum we noticed the absence of the singlet at δ 191.6, assigned to the α,β-unsaturated ketone group (O = C-7) in **1**, the presence of a doublet at δ 74.0, assigned to a secondary oxygenated carbon (C-7), and the upfield ($\Delta\delta$ 18.9) and the downfield shift ($\Delta\delta$ 7.4) of C-14 and C-6 at δ 133.7 and 111.0, respectively.

These findings suggested the structure **2** for sphaeropsidin B. The structure was supported by the EIMS results. In fact, the molecular ion at *m/z* 348 losing in succession H_2O, CO_2 and Me could account for the ions at *m/z* 330, 286 and 271, respectively. By an alternative fragmentation mechanism the ion at *m/z* 330 losing in succession H_2O and Me or CO_2 and OH could account for the ions at *m/z* 312 and 297 or 269, respectively. Finally, loss of a CO molecule from the molecular ion could produce the ion at *m/z* 320. Its FAB mass spectrum showed the pseudomolecular ion $[MH]^+$ at *m/z* 349.

The structure **2** proposed for B was confirmed by chemical work. By reaction with Corey's reagent [3] **2** was oxidized to **1**, while the $NaBH_4$ reduction of the ketone group at C-7 allowed to convert **1** into **2**. As previously described [4], the last reaction afforded only one epimer at C-7 that corresponded to **2**. In conclusion, sphaeropsidin B may be formulated as the 9H-10,4a-(epoxymethane)-phenanthrene-12-one-7-ethenyl-1,2,3,4,4b,5,6,7,10,10a-decahydro-4b,9-dihydroxy-1,1,7-trimethyl.

Sphaeropsidin B should be identical to the metabolite with antibiotic and antiprotozoal activity previously isolated from *Aspergillus chevalieri* and labelled as LL-S491γ. In fact, compound **2** showed physical ($[\alpha]_D$ and mp) and spectral properties very similar to those partially reported for the antibiotic LL-S491γ [4]. This result is not surprising since sphaeropsidin A, the main phytotoxic metabolite recently isolated by our group from *S. sapinea* f.s. *cupressi*, was shown to be identical to the other antibiotic (LL-S491β) produced from the same fungus [2].

Table 3. ^{1}H NMR data of sphaeropsidins A, B and C (**1**, **2**, and **3**, respectively). The chemical shifts are in δ-values (ppm) from TMS*

H	1†	2‡	3‡
1	2.22 *br d* (10.7)	2.05 *br d* (9.8)	2.18 *br d* (13.1)
1′	1.58 *m*	1.46 *m*	1.57 *m*
2	1.58 *m*	1.46 *m*	1.84 *m*
2′	1.58 *m*	1.46 *m*	1.58 *m*
3	1.86 *m*	1.87 *ddd* (15.5, 15.5, 5.2)	1.86 *m*
3′	1.82 *m*	1.46 *m*	1.63 *dd* (14.7, 11.6)
5	2.70 *s*	2.53 *s*	2.33 *dd* (12.8, 6.6)
6	—	—	2.69 *dd* (18.7, 12.8)
6′	—	—	2.46 *dd* (18.7, 6.6)
7	—	4.22 *d* (2.4)	—
11	1.35 *br d* (14.1)	1.23 *br d* (12.8)	1.42 *m*
11′	1.18 *m*	1.12 *m*	1.29 *td* (13.4, 4.0)
12	1.84 *m*	1.72 *dd* (12.8, 2.4)	1.80 *m*
12′	1.64 *m*	1.63 *ddd* (12.8, 3.5, 2.4)	1.50 *m*
14	6.82 *br s*	5.81 *dd* (2.4, 2.4)	6.58 *brs*
15	5.83 *dd* (17.6, 10.5)	5.78 *dd* (17.3, 10.5)	5.78 *dd* (17.5, 10.7)
16	5.06 *dd* (17.6, 1.5)	4.96 *dd* (17.3, 1.0)	5.00 *d* (17.5)
16′	5.06 *dd* (10.5, 1.5)	4.90 *dd* (10.5, 1.0)	4.95 *d* (10.7)
17	1.06 *s*	0.91 *s*	0.94 *s*
18[a]	1.17 *s*	1.14 *s*	0.82 *s*
19[a]	1.16 *s*	1.04 *s*	0.75 *s*

* The coupling constants (J) reported in parentheses are in Hz

† The spectrum was recorded in $CDCl_3$: the signal of the two hydroxy group both braod singlet appeared at δ 5.20 (HO-6) and 2.18 (HO-9).

‡ 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.

[a] These assignments may be reversed.

Sphaeropsidin C (**3**) showed a molecular formula of $C_{20}H_{28}O_4$, as deduced from its HR-EI mass spectrum, for a total of seven unsaturations. The IR spectrum exhibited the presence of the bands of hydroxy, carboxylic, conjugated carbonyl and olefinic groups [5]; the UV spectrum showed the presence of a maximum, typical of an α,β-unsaturated ketone, at 244 nm [6]. Its ^{1}H NMR spectrum (Table 3) differed from that of **1** essentially by the absence of the singlet of H-5 resonating in **1** at δ 2.70 and by the presence of the signals of an ABX system. In fact, a double doublet ($J_{5,6}$ = 12.8 and $J_{5,6'}$ = 6.6 Hz), assigned to the X part (H-5), was observed at δ 2.33 while the AB part appeared as two double doublets ($J_{5,6}$ = 12.8 and $J_{6,6'}$ = 18.7 Hz, and $J_{5,6'}$ = 6.6 and $J_{6,6'}$ = 18.7 Hz, respectively) centred at δ 2.69 and 2.46 (H-6 and H-6′). A broad singlet typical of an acid proton was observed at δ 12.2 when the ^{1}H NMR spectrum was recorded in DMSO-d_6 [7]. The ^{13}C NMR spectrum (Table 4), compared to that of **1**, showed the significant absence of the quaternary carbon at δ 103.6, due to the hemiacetal lactonized carbon in **1**, the presence of a further methylene group resonating at δ 37.1 (C-6) and the significant upfield shift ($\Delta\delta$ 8.8) of the secondary joint ring carbon (C-5) at δ 42.4. Furthermore, the singlet of the carboxylic group (HOOC-20) was observed at a typical chemical shift value of δ 177.6 [8].

Extensive 1D and 2D NMR experiments, namely COSY-45, TOCSY (Correlated and Total Correlation Spectroscopy) [9, 10], HMQC (Heteronuclear Multiple Quantum Correlation) [11] allowed the assignment of all protons and the corresponding carbons (Tables 3 and 4). The results of HMBC (Heteronuclear Multiple-Bond Correlation) [12] (Table 5) and NOESY (Nuclear Overhauser Effect Spectroscopy) [13] (Table 6) experiments allowed assignment of structure **3** to sphaeropsidin C, which revealed an unrearranged diterpenoid also for this toxin.

The structure assigned to sphaeropsidin C was supported by the examination of its HR-EI mass spectrum. As expected the molecular ion at m/z 332.1948 ($C_{20}H_{28}O_4$), losing in succession H_2O, CO_2 and Me residues, would yield the ions at m/z 314.1942 ($C_{20}H_{26}O_3$), 270 and 255, respectively. By an alternative fragmentation mechanism the ion at m/z 314.1942, losing in succession Me and CO, would account for the ions at m/z 299 and 271, respectively. Finally, the molecular ion by the alternative losses of OH or H_2O followed by CO molecules could generate the ions at m/z 315 and 286, respectively [7]. Its FAB mass spectrum showed the pseudomolecular ion $[MH]^+$ at m/z 333, which losing H_2O yielded the ion at m/z 315.

The structure **3** assigned to sphaeropsidin C was confirmed by preparing two key derivatives.

Table 4. ^{13}C NMR data of sphaeropsidins A, B and C (**1**, **2** and **3**, respectively). The chemical shifts are in δ-values (ppm) from TMS*

C	1	2†	3†
1	22.8 *t*	22.8 *t*	27.1 *t*[a]
2	17.9 *t*	18.3 *t*	19.5 *t*
3	26.7 *t*	27.5 *t*	27.6 *t*[a]
4	32.2 *s*	31.8 *s*	33.5 *s*
5	51.2 *d*	51.5 *d*	42.4 *d*
6	103.6 *s*	111.0 *s*	37.1 *t*
7	191.6 *s*	74.0 *d*	201.1 *s*
8	132.9 *s*	135.0 *s*	136.5 *s*
9	70.9 *s*	71.1 *s*	71.7 *s*
10	57.0 *s*	58.1 *s*	52.9 *s*
11	40.3 *t*	40.5 *t*	41.2 *t*
12	29.3 *t*	30.1 *t*	29.4 *t*
13	39.3 *s*	37.8 *s*	38.3 *s*
14	152.6 *d*	133.7 *d*	144.8 *d*
15	144.2 *d*	147.2 *d*	145.8 *d*
16	113.3 *t*	111.0 *t*	111.9 *t*
17	24.3 *q*	23.7 *q*	23.4 *q*
18[a]	32.5 *q*	32.3 *q*	31.1 *q*
19[a]	22.3 *q*	21.8 *q*	19.2 *q*
20	174.7 *s*	176.7 *s*	177.6 *s*

* Multiplicites were determined by DEPT spectrum.

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

[a] These assignments may be reversed.

Reduction of **3** with $NaBH_4$ gave the corresponding secondary alcohol **4**, which probably was stereospecifically formed as mentioned above to support the stereoselective transformation of **1** into **2** [4]. The reduced derivative **4** (*m/z* 334 by EIMS) showed the expected hydroxy, carboxylic and olefinic bands in its IR spectrum [5], while the UV spectra had only an end absorption. Its ^1H NMR spectrum showed, with respect to that of **3**, the presence of the doublet of double doublets ($J_{6,7}$ = 10.6, $J_{6',7}$ = 6.8, and $J_{7,14}$ = 2.0 Hz) due to H-7 at δ 4.29 and the upfield shift ($\Delta\delta$ 0.98) of H-14, which appeared as a double doublet ($J_{7,12} = J_{7,14}$ = 2.0 Hz) at δ 5.60 and that of the signals due to the HC(5)-CH_2(6) fragment which resonated as more complex systems between δ 2.20 and 1.20. The ^{13}C NMR spectrum differed from that of **3**, by the absence of the signal of the ketone carbonyl and for the presence of that of a secondary hydroxylated carbon (HOCH-7) at δ 68.5 and the significant upfield shift ($\Delta\delta$17.4) of C-14 at δ 127.4.

By reaction with diazomethane, **3** gave the corresponding methyl ester derivative **5** that also showed, in agreement to ref. [4], the conversion of the double bond of the α,β-unsaturated ketone into a cyclopropane ring. Derivative **5** (*m/z* 360 by EIMS) showed in the IR spectrum the presence of band typical of hydroxy, carbonyl and olefinic groups [5] and had only an end absorption in the UV spectrum. Its ^1H NMR spectrum showed the expected singlet of the

Table 5. 2D ^{13}C, ^1H long-range correlations measured in the HMBC spectrum carried out on sphaeropsidin C (**3**)

C	H	C	H
27.1 (C-1)	1.42 (H-11)	41.2 (C-11)	2.18 (H-1)
19.5 (C-2)	1.57 (H-1')	29.4 (C-12)	6.58 (H-14), 5.78 (H-15), 5.00 (H-16) 4.95 (H-16'), 0.94 (Me-17)
27.6 (C-3)	1.84 (H-2)	38.3 (C-13)	6.58 (H-14), 5.78 (H-15), 5.00 (H-16) 4.95 (H-16'), 1.80 (H-12), 1.50 (H-12')
33.5 (C-4)	2.69 (H-6), 2.33 (H-5), 1.58 (H-2'), 0.82 (Me-18), 0.75 (Me-19)	144.8 (C-14)	5.78 (H-15), 1.50 (H-12'), 1.42 (H-11), 0.94 (Me-17)
42.4 (C-5)	2.69 (H-6), 2.46 (H-6'), 2.18 (H-1), 1.42 (H-11), 0.82 (Me-18), 0.75 (Me-19)	145.8 (C-15)	6.58 (H-14), 5.00 (H-16) 4.95 (H-16'), 1.80 (H-12), 0.94 (Me-17)
37.1 (C-6)	2.33 (H-5)	23.4 (C-17)	5.78 (H-15), 1.80 (H-12)
201.1 (C-7)	6.58 (H-14), 2.69 (H-6), 2.46 (H-6')	31.1 (C-18)	0.75 (Me-19)
136.5 (C-8)	6.58 (H-14), 1.80 (H-12)	19.2 (C-19)	2.46 (H-6'), 0.82 (Me-18)
71.7 (C-9)	6.58 (H-14), 2.33 (H-5) 1.80 (H-12), 1.57 (H-1'), 1.50 (H-12')	177.6 (C-20)	2.33 (H-5), 2.18 (H-1), 1.58 (H-2')
52.9 (C-10)	2.46 (H-6), 2.33 (H-5), 2.18 (H-1), 1.58 (H-2')		

Table 6. 2D ^{1}H NOE (NOESY) data obtained for sphaeropsidin C (3)

Considered	Effects
6.58 (H-14)	1.80 (H-12), 1.50 (H-12′)
2.33 (H-5)	2.69 (H-6), 2.46 (H-6′), 1.86 (H-3), 1.58 (H-2′), 0.82 (Me-18)
2.18 (H-1)	1.58 (H-2′)
1.80 (H-12)	5.78 (H-15), 5.00 (H-16)
1.50 (H-12′)	5.78 (H-15), 5.00 (H-16)
1.29 (H-11′)	1.57 (H-1′)
0.94 (Me-17)	5.78 (H-15), 5.00 (H-16), 1.80 (H-12), 1.50 (H-12′)
0.82 (Me-18)	2.69 (H-6), 2.46 (H-6′), 2.33 (H-5), 1.58 (H-2′)
0.75 (Me-19)	2.69 (H-6), 2.46 (H-6′), 1.84 (H-2)

methyl ester group at δ 3.65, a double doublet ($J_{14,21} = J_{14,21'} = 8.8$ Hz) and a complex system, due to the H-14 proton at the junction and the methylene (H_2C-21) of the cyclopropane ring at δ 2.10 and 1.07, respectively. The ^{13}C NMR spectrum differed from that of **3** by the absence of the signals of the trisubstituted olefinic group of the α,β-unsaturated ketone, the downfield shift ($\Delta\delta$ 7.4) of a ketone carbonyl signal at δ 208.5, the presence of the methoxy carbon and the signals of the 1,1,2-trisubstituted cyclopropane ring at δ 52.2 (OMe) and 34.3 (C-14), 29.2 (C-8) and 22.7 (C-21).

Finally, the structure **3** deduced from HR-EIMS and NMR data, has been confirmed by an X-ray study (unpublished results). Therefore, sphaeropsidin C was found to be a new tricyclic pimarane diterpene having an acid nature. It can be formulated as 4a(9*H*)-phenanthrenecarboxylic acid, 9-one-7-ethenyl-1,2,3,4,4b,5,6,7,10,10a-decahydro-4b-hydroxy-1,1,7-trimethyl.

Sphaeropsidins B and C, as well as the recently reported sphaeropsidin A (**1**), belong to unrearranged pimaranes, a group of diterpenes already known as metabolites of plant, microorganism and marine organisms some of which show interesting biological activity [14–17]. To the best of our knowledge, sphaeropsidin B has been isolated for the first time as a phytotoxin and as metabolite of *S. sapinea* f.s. *cupressi*. However, as judged from all the above data **2** is identical to the antibiotic labelled as LL-S491γ previously isolated from *Aspergillus chevalieri* [4, 15]. Furthermore, for the first time sphaeropsidin A was identified as the main phytotoxin of *D. mutila*. Sphaeropsindin C represents a new phytotoxic fungal metabolite which differs from the already known acid unrearranged pimaranes by the location of the carboxylic group and the functionalization of the phenanthrene ring system [14].

EXPERIMENTAL

General. Mps uncorr.; optical rotations: MeOH; IR and UV: neat and MeOH, respectively; ^{1}H and ^{13}C NMR: 500, 400 or 270 and 125, 100 or 67.92 MHz, respectively, in $CDCl_3$–CD_3OD (95:5), unless otherwise noted, using $CDCl_3$ as int. standard. Carbon multiplicities were determined by DEPT (Distortionless Enhancement by Polarization Transfer) spectra [8]. DEPT, TOCSY, HMQC, HMBC and NOESY NMR experiments were performed using Bruker microprograms. EI and HR-EIMS: 70 eV; FABMS: glycerol–thioglycerol using Cs as bombarding atoms. Analytical and preparative TLC: silica gel (Merck, Kieselgel, 60 F_{254}, 0.25 and 0.50 mm, respectively) or on reverse phase (Whatman, KC18 F_{254}, 0.20 mm) plates; the 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 system: (A) $CHCl_3$–*iso*-PrOH (19:1); (B) petrol–Me_2CO (2.3:1); (C) EtOH–H_2O (1.5:1). The petrol used had bp 40–70°.

Fungi species. Sphaeropsis sapinea f. sp. *cupressi* and *Diplodia mutila* were isolated from infected cypress (*Cupressus sempervirens* L.) trees from Morocco and from infected cypress (*C. sempervirens*) and oak (*Quercus cerri*, *Q. ilex* and *Q. robur*) trees from Southern and Northern Italy, respectively, identified by Prof. S. Frisullo (Universitá di Bari, Foggia) and deposited in the fungal collection of the 'Dipartimento di Patologia Vegetale, Universitá di Bari', Italy (N. 1524 and 1602, respectively).

Production and bioassays of sphaeropsidins. The methods used for the prepn of cultures of *S. sapinea* f. sp. *cupressi* and *D. mutila* as well as for the assay of phytotoxicity of sphaeropsidins B and C on host (cypress: *Cupressus sempervirens*, *C. macrocarpa* and *C. arizonica*; oak: *Quercus cerri*, *Q. ilex* and *Q. robur*) and non-host (*Lycopersicon esculentum* cv. Marmande, *Avena sativa* cv. Park) seedlings or cuttings were those presented in a recent paper [2]. The antifungal activity of both toxins has been checked on several phytopathogenic fungal species: *Botrytis cinerea*, *Colletotrichum acutatum*, *Fusarium*) *oxysporum*, *Phomopsis* (*Fusicoccum*) *amygdali*, *Sclerotinia sclerotiorum*, *Seiridium cardinale* and *S. cupressi* as already described in detail [2].

Extraction and purification of sphaeropsidins. The culture filtrates (10 l) of *S. sapinea* f.s. *cupressi* obtained as above described were acidified to pH 4 with 2 M HCl and extracted with EtOAc (4 × 5 l). The combined organic extracts were dried (Na_2SO_4) and evapd under red. pres. to give a red-brown oil residue (775 mg) having an high phytotoxic activity. TLC analysis (silica gel, eluent system A) of this latter showed the presence of sphaeropsidin A (**1**) at R_f 0.65 and that of other three main metabolites at R_f 0.50, 0.43 and 0.38, respectively. The crude residue was chromatographed by CC eluted with solvent system A to afford 9 groups of homogeneous frs, of which groups 2–7 showed phytotoxic activity. The residues (191 mg) left from fr. groups 3 and 4 were combined

and purified in the conditions described above to yield sphaeropsidin A (**1**) (131 mg), which crystallized from EtOAc–*n*-hexane as colorless needles (120 mg), and three frs containing a residual amount of **1** and the other two more polar metabolites (R_f 0.50, 0.43). The residues of these latter, and that of the mother liquors of sphaeropsidin A crystallization, were combined (49.7 mg) and further purified by two succesive prep. TLC steps (silica gel, solvent system A). This process gave, as amorphous solids, further amounts of sphaeropsidin A (4.5 mg) and the other two metabolites, named sphaeropsidin C (**3**, 9.3 mg, R_f 0.50 and 0.30, by silica gel and reverse phase TLC, eluent systems A and C, respectively) and sphaeropsidin B (**2**, 28.8 mg, R_f 0.43, and 0.46 and 0.30, by silica gel and reverse phase TLC, eluent systems A, B and C, respectively). The residue (355 mg) left from fr. groups 5–7 of the initial column was further purified by prep. TLC as above described to afford further amounts of sphaeropsidins A, B and C (15.5, 51.2 and 15.7 mg, for a total 14.0, 8.0 and 2.5 mg l^{-1}, respectively). Both sphaeropsidins B and C crystallized from $CHCl_3$ MeOH as colorless needles.

Extraction and fractionation of *D. mutila* culture filtrates (1.5 l) by the same procedure resulted in the main phytotoxin (40 mg, 27.0 mg/l) and sphaeropsidin C (14 mg, 9.3 mg l^{-1}), both obtained as crystalline solids. The first showed the same physical and spectroscopic properties recently reported for **1** [2] and was identified as sphaeropsidin A.

Sphaeropsidin B (**2**). Colorless needles: mp 185–190°; $[\alpha]_D^{25}$ +63 (*c* 0.8). UV λ_{max} nm (logε): < 220. IR ν_{max} cm^{-1}: 3405, 1737, 1642. ^1H NMR and ^{13}C NMR: Tables 3 and 4, respectively; [lit. 4: mp 190–195°; $[\alpha]_D$ +69.3 (MeOH). UV: end absorption. IR ν_{max}^{KBr} cm^{-1}: 1745. ^1H NMR ($CDCl_3$): δ 5.83 and 4.30 (1H and 1H, $J_{7,14}$ = 2.0 Hz, H-14 and H-7, respectively)]. EIMS *m/z* (rel. int.): 348 $[M]^+$ (1.5), 330 $[M\text{-}H_2O]^+$ (7), 320 $[M\text{-}CO]^+$ (4), 312 $[M\text{-}2\times H_2O]^+$ (7), 297 $[M\text{-}2\times H_2O\text{-}Me]^+$ (5), 286 $[M\text{-}H_2O\text{-}CO_2]^+$ (50), 271 $[M\text{-}H_2O\text{-}CO_2\text{-}Me]^+$ (78), 269 $[M\text{-}H_2O\text{-}CO_2\text{-}OH]^+$ (22), 166 (73), 151 (68), 133 (100), 105 (95), 91 (85), 77 (64), 67 (61), 55 (72), 41 (64). FABMS (+) *m/z* (rel. int.): 349 $[MH]^+$ (100).

Sphaeropsidin C (**3**). Colorless needles: mp 250–255°; $[\alpha]_D^{25}$ +16.8 (*c* 1.0). UV λ_{max} nm (logε): 244 (3.74). IR ν_{max} cm^{-1}: 3245, 1723, 1678, 1609. ^1H NMR and ^{13}CNMR: Tables 3 and 4, respectively. HR-EIMS *m/z* (rel. int.): 332.1948 (calcd for $C_{20}H_{28}O_4$, 332.1980, 5) $[M]^+$, 315 $[M\text{-}OH]^+$ (16), 314.1942 (calcd for $C_{20}H_{26}O_3$, 314.1882, 20) $[M\text{-}H_2O]^+$, 299 $[M\text{-}H_2O\text{-}Me]^+$ (10), 286 $[M\text{-}H_2O\text{-}CO]^+$ (18), 271 $[M\text{-}H_2O\text{-}Me\text{-}CO]^+$ (27), 270 $[M\text{-}H_2O\text{-}CO_2]^+$ (48), 255 $[M\text{-}H_2O\text{-}CO_2\text{-}Me]^+$ (54), 178 (100), 163 (95), 149 (95) 135 (90), 121 (90), 91 (90), 77 (82), 55 (80). FABMS (+) *m/z* (rel. int.) 333 $[MH]^+$ (37), 315 $[MH\text{-}H_2O]^+$ (100).

$NaBH_4$ *reduction of sphaeropsidin C*. Sphaeropsidin C (**3**, 10 mg) in MeOH (5 ml) was treated with $NaBH_4$ (10 mg) with stirring at room temp for 30 min. The mix. was neutralized with 0.1M HCl and, extracted with CH_2Cl_2 (4 × 10 ml) and dried. The oily residue (8.7 mg) was purified by prep. TLC (silica gel, solvent system A) to give the dihydroderivative **4** as a homogeneous compound (5 mg): UV λ_{max} nm (logε): <220; IR ν_{max} cm^{-1}: 3320, 1724, 1680. The ^1H NMR and ^{13}C NMR spectra differed from those of **3** in the following signals: ^1H NMR: δ 5.60 (1H, *dd*, $J_{7,12} = J_{7,14}$ = 2.0 Hz, H-14), 4.29 (1H, *ddd*, $J_{6,7}$ = 10.6, $J_{6',7}$ = 6.8, and $J_{7,14}$ = 2.0 Hz, H-7); ^{13}C NMR: δ 127.4 (*d*, C-14), 68.5 (*d*, C-7). EIMS *m/z* (rel. int.): 334 $[M]^+$ (2), 317 $[M\text{-}OH]^+$ (1), 316 $[M\text{-}H_2O]^+$ (4), 300 $[M\text{-}2\times OH]^+$ (2), 299 $[M\text{-}OH\text{-}H_2O]^+$ (7), 298 $[M\text{-}2\times H_2O]^+$ (30) 284 $[M\text{-}OH\text{-}H_2O\text{-}Me]^+$ (13) 283 $[M\text{-}2\times H_2O\text{-}Me]^+$ (61), 272 $[M\text{-}H_2O\text{-}CO_2]^+$ (13), 270 $[M\text{-}2\times H_2O\text{-}CO]^+$ (4), 257 $[M\text{-}H_2O\text{-}CO_2\text{-}Me]^+$ (9), 255 $[M\text{-}2\times H_2O\text{-}CO\text{-}Me]^+$ (8), 242 $[M\text{-}H_2O\text{-}CO_2\text{-}2\times Me]^+$ (23), 162 (100), 147 (95), 120 (84), 105 (83), 91 (75), 55 (69). FABMS (+) *m/z* (rel. int.): 317 $[MH\text{-}H_2O]^+$ (100), 299 $[MH\text{-}2\times H_2O]^+$ (86), 271 $[MH\text{-}2\times H_2O\text{-}CO]^+$ (86).

Reaction of sphaeropsidin C with diazomethane. Ethereal CH_2N_2 (1.5 ml) was added, at 0°, to **3** (10 mg) in MeOH (5 ml). The mix. was allowed to stand at room temp for 1 hr and was then evap under a N_2 stream. Purification of the residue by prep TLC (silica gel, solvent system A) produced the main derivative corresponding to the methyl ester **5** (4.5 mg). UV λ_{max} nm (logε): <220 nm. IRν_{max} cm^{-1}: 3415, 1720, 1671. The ^1H NMR and ^{13}C NMR spectra ($CDCl_3$) differed from those of **3** in the following signal systems: ^1H NMR: δ 3.65 (3H, *s*, OMe), 2.10 (1H, *dd*, $J_{14,21} = J_{14,21'}$ = 8.8 Hz, H-14), 1.07 (2H, *m*, H-21 and H-21′); ^{13}C NMR: δ 208.5 (*s*, C-7), 52.2 (*q*, OMe), 34.3 (*d*, C-14), 29.2 (*s*, C-8), 22.7 (*t*, C-21). EIMS *m/z* (rel. int.): 360 $[M]^+$ (3), 343 $[M-OH]^+$ (2), 342 $[M-H_2O]^+$ (6), 328 $[M-MeOH]^+$ (11), 310 $[M-MeOH-H_2O]^+$ (6), 300 $[M-MeOH-CO]^+$ (11), 283 $[M-H_2O-MeCOO]^+$ (10), 192 (100), 177 (94), 149 (88), 138 (95), 124 (86), 109 (79), 81 (77), 55 (74). FABMS (+) *m/z* (rel. int.): 361 $[MH]^+$ (100), 343 $[MH-H_2O]^+$ (95), 329 $[MH-MeOH]^+$ (50).

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