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Chemical and biological characterisation of sapinopyridione, a phytotoxic 3,3,6-trisubstituted-2,4-pyridione produced by *Sphaeropsis sapinea*, a toxigenic pathogen of native and exotic conifers, and its derivatives

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Abstract

A phytotoxic trisubstituted 2,4-pyridione, named sapinopyridione, was isolated from the culture filtrates of *Sphaeropsis sapinea*, a fungal pathogen of conifers occurring world-wide. Three strains were isolated from two cypress species. Strain D-55 isolated from *Cupressus sempervirens* resulted high producer of sapinopyridione (12.3 mg l^{-1}), whereas strain D-54 isolated from the same cypress species was low producer (1.1 mg l^{-1}); strain D-50 isolated from *C. macrocarpa* was intermediate producer (5.4 mg l^{-1}). Sapinopyridione was characterised by spectroscopic and chemical methods, as the 6-methyl-2-(2-methyl-1-oxobutyl)-1-oxa-5-azaspiro[2.5]oct-6-ene-4,8-dione. The structure was supported by the preparation of three key derivatives, whose phytotoxic and antimycotic activities were also tested on host plants and on three *Seiridium* species, virulent fungal agents of cypress canker disease. Some structure–activity relationships were identified for both phytoxicity and antifungal activities. These activities appear related to the presence of both pyridione and oxiran rings. Also the carbonyl group of the side chain seems to play a role into impart activity.

Keywords: *Sphaeropsis sapinea*; Conifer pathogen; Phytotoxins; 3,3,6-Trisubstituted-2,4-pyridione; Sapinopyridione derivatives; Bioactivity; Structure–activity relationship

1. Introduction

Sphaeropsis sapinea (Fr.:Fr.) Dyko & Sutton [syn. *Diplodia pinea* (Desmaz.) J. Kickx f.] is an opportunistic plant pathogen showing a cosmopolitan distribution and an extensive host range on conifers. The pathogen occurs in coniferous forests throughout the world and has been associated with significant economic damages in exotic plantations in New Zealand, Australia, and South Africa (Chou, 1976; Zwolinski et al., 1990). *S. sapinea* also occurs in the central and eastern United States causing severe damage on both native and introduced species (Palmer et al., 1987). Symptoms include post-hail associated dieback, cankers, crown wilt, shoot blight, root disease and collar rot (Gibson, 1979; Chou, 1987; Swart and Wingfield, 1991; Stanosz and Cummings Carlson, 1996). Dark blue to black staining also results from colonisation of sapwood, either in standing trees or recently harvested logs (Chou, 1976, 1987; Celimene et al., 2001).

The taxonomy of *S. sapinea* has been the subject of considerable confusion and conflicting reports (Chou, 1976, 1987; Swart and Wingfield, 1991). Initially two morphotypes, A and B, were described for *S. sapinea* and these

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Nomenclature

Sapinopyridione (**1**): 6-methyl-2-(2-methyl-1-oxobutyl)-1-oxa-5-azaspiro[2.5]oct-6-ene-4,8-dione

9,*O*-Dihydrosapinopyridione (**2**): 6-methyl-2-(1-hydroxy-2-methylbutyl)-1-oxa-5-azaspiro[2.5]oct-6-ene-4,8-dione

4-Hydroxy-6-methyl-2-pyridone derivative (**3**): 3-*O*-(3-methyl-2-oxo-)pentyl-4-hydroxy-6-methyl-2-pyridone

4-Hydroxy-6-methyl-2-pyridone derivative (**4**): 3-(1-hydroxy-3-methyl-2-oxo)pentyl-4-hydroxy-6-methyl-2-pyridone

were defined based on cultural characteristics, texture of conidial walls and virulence (Wang et al., 1985; Palmer et al., 1987). The distinction between A and B morphotypes of *S. sapinea* has been confirmed using randomly amplified polymorphic DNA (RAPDs), sequences from the ITS region of the rRNA operon and conidial size (Smith and Stanosz, 1995; Stanosz et al., 1996, 1999; de Wet et al., 2000), restriction fragment length polymorphisms (RFLPs) (Hausner et al., 1999). A third RAPD group, including isolates from Indonesia and Mexico, has recently been reported and was designated as C morphotype based on differences in conidial size (de Wet et al., 2000). Isolates of A morphotype were aggressive on both jack pine and red pine, but B morphotype isolates caused severe symptoms only on jack pine (Blogget and Stanosz, 1997). Moreover, C morphotype isolates artificially inoculated on both Granny Smith apples and *Pinus patula* seedlings revealed that isolates of C morphotype were considerably more virulent than those of the A and B morphotypes (de Wet et al., 2002). More recently, de Wet et al. (2003) provided strong evidence that the B morphotype isolates are distantly related to *S. sapinea* and represent a discrete taxon, designated as *Diplodia scrobiculata* sp. nov.

The isolation and the identification of secondary metabolites could contribute to better characterise the biology, the physiology and the pathogenicity of *S. sapinea* morphotypes. Moreover, the possibility of host specialisation of these groups could be explained.

Recently, we had undertaken a study on the production and identification of secondary metabolites produced by three strains of *S. sapinea*, isolated from infected cypress trees. The two main metabolites, named sapinofuranones A and B characterised as two epimeric 2(3H)-dihydrofuranones, proved to be phytotoxic on non-host plants such as tomato and on host plants as cypress and pine (Evidente et al., 1999). As expected, the two sapinofuranones were chemically different from the phytotoxins (sphaeropsidins A–F) produced in vitro by *S. sapinea* f. sp. *cupressi* and *D. mutila* isolated from the same cypress plants colonised by *S. sapinea* strains and responsible of a different form of cypress canker (Frisullo et al., 1997; Sparapano et al., 2004).

The objective of this study was to fully elucidate the structure of a new phytotoxic 3,3,6-trisubstituted-2,4-pyridione, referred as sapinopyridione (**1**), by means of NMR and MS techniques. This structure was confirmed by preparation of three key derivatives (**2**–**4**). A structure–activity relationship study was also undertaken through the bioactivity elucidation of sapinopyridione and its derivatives assessed by phytotoxic and antimycotic bioassays.

2. Results and discussion

The crude organic extract of culture filtrate of all three strains (D-50, D-54 and D-55) of *S. sapinea*, showing a high phytotoxicity on both host and non-host plants, was purified by combination of column and TLC chromatography to give the main toxic metabolites, sapinofuranones A and B contained in the fraction 5 obtained by the initial column chromatography (Evidente et al., 1999). The purification of the subsequent fraction 8, using the same chromatographic procedure as described in detail in Section 3, allowed to isolate a minor metabolite obtained as white needles. The preliminary spectroscopic investigation (^{1}H and ^{13}C NMR and MS) proved that this latter substantially differed from sapinofuranones and on the basis of the structural characteristics, decribed below, it was named sapinopyridione (**1**, Fig. 1).

Sapinopyridione has a molecular formula of $C_{12}H_{15}NO_4$, corresponding to six degrees of unsaturation, as deduced from the molecular weight of 237.1012, measured by HR EI mass spectrometry. The investigation of its ^{1}H NMR spectrum recorded in $CDCl_3$ (Table 1) showed a broad singlet at δ 8.38 (NH), a typical chemical shift value

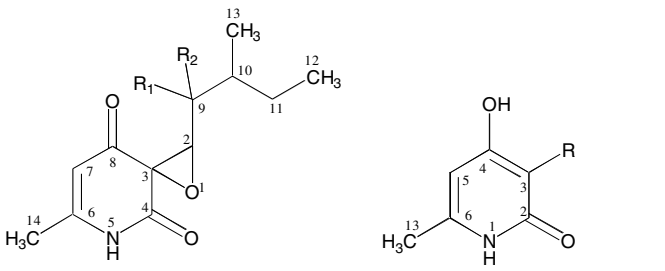

Fig. 1. Structures of sapinopyridione (**1**) and its derivatives (**2**–**4**).

Table 1
^{1}H and ^{13}C NMR data of sapinopyridione (**1**)[a]

C	δC[b]	δH	J (Hz)	HMBC
2	68.6 (*d*)	3.94 (*s*)		
3	59.9 (*s*)			
4	168.2 (*s*)			
6	154.9 (*s*)			5.64, 2.19
7	107.1 (*d*)	5.64 (*s*)		
8	186.2 (*s*)			3.94
9	206.5 (*s*)			3.28, 1.79, 1.52, 1.05
10	44.3 (*d*)	3.28 (*ddq*)	7.7, 6.4, 4.9	1.79, 1.52, 1.05, 0.97
11	25.3 (*t*)	1.79 (*ddq*)	14.7, 7.4, 4.9	3.28, 1.05, 0.97
		1.52 (*ddq*)	14.7, 7.7, 7.4	
12	11.1 (*q*)	0.97 (3H, *t*)	7.4	3.28, 1.79, 1.52
13	12.6 (*q*)	1.05 (3H, *d*)	6.4	3.28, 1.79, 1.52
14	20.8 (*q*)	2.19 (3H, *s*)		
NH		8.38 (*br s*)		

The chemical shift are in δ-values (ppm) from TMS.
[a] 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.
[b] The multiplicities were determined by DEPT spectra.

of a lactam proton (Pretsch et al., 1989), which in the COSY and TOCSY spectra (Berger and Braun, 2004) coupled with the singlet of an olefinic proton resonating at δ 5.64 (H-7) and this latter ($J < 1$ Hz) in turn with a methyl group at δ 2.19 (Me-14) (Pretsch et al., 1989). The latter proved to be linked to the quaternary carbon of the same double bond by the correlation observed in the HMBC spectrum (Berger and Braun, 2004) (Table 1). These findings are in agreement with the signal system observed in the ^{13}C NMR spectrum (Table 1) and the couplings observed in the HSQC spectrum (Berger and Braun, 2004), which allowed to assign to the carbons of the lactam carbonyl, the olefinic and the methyl groups the typical chemical shifts values of δ 168.2, 154.9 and 107.1 and 20.8 (C-4, C-6 and C-7 and C-14, respectively) (Breitmaier and Voelter, 1987). Other two carbons resonated in the same spectrum at δ 206.5 and 186.2 (C-9 and C-8, respectively), typical shift values for a saturated and a conjugated carbonyl group (Breitmaier and Voelter, 1987). These structural features together with the presence of a quaternary carbon (C-3) appearing in the ^{13}C NMR spectrum at δ 59.9 suggested the presence of a 3,3,6-trisubstituted-2,4-pyridione in **1**, with the 3-side chain including the saturated ketone group. These moiety agreed with the typical bands for saturated ketones and α- and γ-pyridones observed in the IR spectrum (Nakanishi and Solomon, 1977) as well as with the observation of a maximum exhibited at 320 nm in the UV spectrum, in agreement with those reported for 4-hydroxy-2-pyridone derivatives (Scott, 1964). The C-3 is a spiro-carbon that belongs to both the 2,4-pyridione and the oxiran ring, whose second C-atom (C-2) is substituted by a 2-methyl-1-oxobutyl side chain. H-2 and its geminal carbon (C-2) appeared, respectively, at δ 3.94 and 68.6 in the ^{1}H and ^{13}C NMR spectrum, typical chemical shifts value of a trisubstituted oxiran ring (Breitmaier and Voelter, 1987; Pretsch et al., 1989). In fact, in the HMBC spectrum H-2 significantly coupled with the O=C-8 carbonyl group. Consequently, the proton and carbon spectra also showed the signal pattern typical of the 2-methyl-1-oxobutyl residue attached to C-2. The assignment of the chemical shift to the all protons and the corresponding carbons of this side chain were also corroborated by the couplings observed in the COSY, TOCSY and HSQC spectra. Therefore, the chemical shifts of all carbons and protons of **1** were summarised in Table 1.

On the basis of data as above mentioned and fully discussed, the structure of a 3,3,6-trisubstituted-2,4-pyridione was assigned to sapinopyridione that can be formulated as the 6-methyl-2-(2-methyl-1-oxobutyl)-1-oxa-5-azaspiro[2.5]oct-6-ene-4,8-dione (**1**, Fig. 1).

The structure was confirmed by the correlation observed in the HMBC spectrum (Table 1) and the MS data. The EIMS spectrum, in addition to the molecular ion at 237.1012, showed the corresponding protonated ion $[M+H]^+$ at *m/z* 238, frequently observed for compounds containing a lactam ring (Pretsch et al., 1989). These two ions generated the two series of peaks at *m/z* 181, 153, 126 and 97 and 180, 152, 125 and 96, by subsequent losses of a C_4H_9, CO, HCN and HCO residues and alternatively the other two series of ions at *m/z* 223 and 195 and 222 and 194 by subsequent losses of Me and CO residues in agreement with the fragmentation mechanism typical of saturated ketones and alkylpyridones (Porter, 1985; Pretsch et al., 1989). Furthermore, the molecular ion by ethylene elimination and in agreement with the typical McLafferty arrangement yielded the ion at *m/z* 209 (Pretsch et al., 1989). As expected, the base peak due to the secondary butyl ion $[C_4H_9]^+$ was observed at *m/z* 57. The (+)ESIMS spectrum showed the potassium $[M+K]^+$ and the sodium $[M+Na]^+$ clusters and the pseudomolecular ions at *m/z* 276, 260 and 238, respectively.

The structure of the toxin is closely related to the toxin produced by an unidentified species of the genus *Macrophoma* causing the fruit rot of apple, a disease frequently observed in orchards of northern Japan. This host-selective toxin named FRT-A, whose structure and absolute configuration was determined by Sassa (1983), was isolated together with the side-chain isomer flavipucine (Sassa and Onuma, 1983). The latter is a well known antibiotic previously isolated from a strain of *Aspergillus flaviceps*, whose stereo-structural characterisation was extensively studied also through the synthesis and X-ray analysis (Findlay and Radics, 1972; Findlay and Kwan, 1972; Peter et al., 1978; Girotra and Wendler, 1979).

However, when the optical rotation of the sapinopyridione was compared with that of FRT-A measured in the same conditions, it appears quite identical in the value but opposite in the sign. This result suggests that the two toxins are enantiomers. This opinion is supported by the fact that other spectroscopic properties are very similar (Sassa, 1983; Sassa and Onuma, 1983). In fact, sapinopyridione showed IR, UV and ^{1}H and ^{13}C NMR data very similar to that of FRT-A as the little observed differences

are due to the different solvent and the more high resolution used in recording the UV and ^{1}H NMR spectra, respectively. Also the mass spectral data including the diagnostic fragmentation peaks are very similar. The absolute configuration of FRT-A toxin was determined by chemical degradation and conformational analysis of its partially reduced derivative at C-1 of the 2-methyloxobutyl side chain. The configuration of the heterocyclic ring of this reduced derivative was deduced by NMR data and confirmed by X-ray crystallographic analysis of synthetic (±)-flavipucine (White et al., 1978), the absolute configuration of the oxiran ring was assigned on the basis of CD data (Sassa, 1983). This stereochemistry is the same in FRT-A, while the absolute configuration of the chiral carbon C-2 of the 2-methyloxobutyl was assigned by chemical degradation (Sassa, 1983). Therefore, the absolute configuration assigned to the FRT-A toxin is 2*S*,3*S*,10*S* (Sassa, 1983). The comparison of the spectroscopic data of the reduced sapinopyridione derivative (**2**), described below, with those of the analogue FRT-A derivative, which appeared to be similar but not inversely, showed they are two diastereomers. This result confirms the enantiomeric nature of the two original toxins. Consequently, the absolute configuration of the sapynopyridione is 2*R*,3*R*,10*R*.

The sapinopyridione, the FRT-A toxin and the flavipucine are the only examples of substituted 2,4-pyridione as naturally occurring compounds. In fact, the 2,4-pyridiones more frequently reported are synthetic hypnotic drugs, which are object of several investigations on their action and metabolism in human and animal cells (Rudy and Senkowski, 1973; Baumeler and Dibach, 1976; Borchert et al., 1982).

S. sapinea strains differed from each other for their yield of toxin production (Table 2). Strain D-50 isolated from *C. macrocarpa* resulted the greatest producer of sapinofuranones A and B (Evidente et al., 1999) and, intermediate producer of **1**. On the contrary, D-55 isolated from *C. sempervirens* was the greatest producer of **1** and the lowest producer of sapinofuranones A and B. The second strain D-54 isolated from *C. sempervirens* resulted the lowest producer of **1** and intermediate producer of sapinofuranones A and B. These findings suggest the fact that there is a gradient of toxin production among the isolates of *S. sapinea* present on both infected cypress species. If we consider the toxins produced and their concentrations as virulence determinants of the pathogen, it is conceivable to assume the existence of virulent (more toxigenic) and hypovirulent (less toxigenic) strains of *S. sapinea* in the population spread over the host plants. These secondary metabolites produced by *S. sapinea* may be implicated in pathogenesis and disease susceptibility.

Furthermore, in order to confirm these findings and to carry out a structure–activity relationship study on the sapinopyridione, three key derivatives were prepared starting from the toxin. The goal of this research was to prepare derivatives modified in the side chain and the pyridione ring.

By $NaBH_4$ reduction, compound **1** was converted into the 9,*O*-dihydroderivative (**2**, Fig. 1): 6-methyl-2-(1-hydroxy-2-methylbutyl)-1-oxa-5-azaspiro[2.5]oct-6-ene-4,8-dione. The reaction carried out as reported by Sassa (1983) for the reduction of FRT-A toxin resulted stereoselective and yielded, as the main product, a diastereomer of the dihydroFRT-A toxin. The reduced derivative of **1** (**2**) showed in the (+)ESI MS spectrum the pseudomolecular and the sodium clustered ions ($[M+H]^+$) and ($[M+Na]^+$) *m*/*z* 240 and 262, respectively and the significant ion at *m*/*z* 222 produced by the pseudomolecular ion by loss of H_2O. In the (−)ESIMS spectrum only the significative pseudomolecular ion ($[M-H]^-$) at *m*/*z* 238 was recorded. The EIMS spectrum showed the molecular ion at *m*/*z* 239 and the fragmentation peaks at *m*/*z* 152, 134 and 123 due to alternative loss of part of side chain [HC(OH)-$CH(CH_3)CH_2CH_3$] followed by H_2O loss or by the total loss of side chain. Alternatively, by successive loss of CO, H_2O, HCN and H_2O molecules, the parent ions generated the peaks at *m*/*z* 211, 193, 166 and 148, respectively. The IR spectrum differed from that of **1** for the significant presence of the bands due to hydroxy groups, while the UV spectra were very similar. The ^{1}H NMR spectra of **2** (Table 3) differed from that of **1** for the presence of a double doublet ($J = 7.0$ and 6.8 Hz) of H-9 at δ 3.80 while the oxiran proton (H-2) and H-10 showed a marked upfield shift ($\Delta\delta$ 0.47 and 1.60, respectively) appearing as a doublet ($J = 7.0$ Hz) and a complex multiplet at δ 3.47 and 1.68, respectively. The ^{13}C NMR spectrum of **2** (Table 4) differed from that of **1** specifically for the absence of the signal of the saturated ketone of the side chain and the presence of the doublet due to the secondary hydroxylated carbon of C-9 at δ 71.2 (Breitmaier and Voelter, 1987). As expected, some spectroscopic properties of **2** were very similar (IR, and MS) to those of the analogue dihydroderivative of the FRT-A toxin but quite different for the UV, CD and partially reported ^{1}H NMR data (Sassa, 1983).

Table 2
Production of sapinopyridione (**1**) in liquid cultures of three strains of *S. sapinea* isolated from *Cupressus macrocarpa* (D-50) or *C. sempervirens* (D-54 and D-55)[a]

Strain	Culture filtrates (l)	Final pH	Mat dry wt (g l^{-1})	Crude extracts (mg l^{-1})	Sapinopyridione (**1**) production (mg l^{-1})
D-50	6.4	6.1 (±0.1)	10.9 (±0.6)	384.4 (±18.1)	5.4 (±1.1)
D-54	7	6.3 (±0.1)	12.6 (±2.8)	267.2 (±25.11)	1.1 (±0.5)
D-55	14	6.8 (±0.1)	7.8 (±0.1)	651.6 (±12.2)	12.3 (±5.5)

[a] Each fungal strain was grown as previously reported in detail (Evidente et al., 1999). Figures are the means ± SD.

Table 3
^{1}H NMR data of sapinopyridione derivatives (**2**–**4**)[a]

H	2		3		4	
	δH	*J* (Hz)	δH	*J* (Hz)	δH	*J* (Hz)
1	–		9.24 (*br s*)		8.96 (*br s*)	
2	3.47 (*d*)	7.0	–		–	
5	10.04 (*br s*)		5.83 (*s*)		5.69 (*s*)	
7	5.62 (1H, *br s*)		4.87 (*d*)	18.7	5.80 (*s*)	
			4.84 (*d*)	18.7		
9	3.80 (*dd*)	7.0, 6.8	2.46 (*ddq*)	7.0, 7.0, 6.9	2.94 (*ddq*)	7.0, 6.9, 6.9
10	1.68 (1H, *m*)		1.68 (*ddq*)	14.0, 7.4, 6.9	1.64 (*ddq*)	14.0, 7.4, 6.9
			1.44 (*ddq*)	14.0, 7.4, 7.0	1.29 (*ddq*)	14.0, 7.4, 7.0
11	1.60 (*m*)		0.86 (3H, *t*)	7.4	0.68 (3H, *t*)	7.4
	1.27 (*m*)					
12	1.00 (3H, *t*)	7.2	1.09 (3H, *d*)	7.0	1.14 (3H, *d*)	6.9
13	1.09 (3H, *d*)	6.8	2.24 (3H, *s*)		2.27 (3H, *s*)	
14	2.39 (*br s*)					
OH	7.6 (*br s*)		10.26 (*br s*)		11.53 (*br s*)	

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

The toxin was converted by catalytic hydrogenation into the two corresponding 3-substituted 4-hydroxy-6-methyl-2-pyridones (**3** and **4**, Fig. 1): 3-*O*-(3-methyl-2-oxo-)pentyl-4-hydroxy-6-methyl-2-pyridone and the 3-(1-hydroxy-3-methyl-2-oxo)pentyl-4-hydroxy-6-methyl-2-pyridone. As expected the two dihydroderivatives showed the same molecular ion at *m/z* 239 in the EIMS spectrum and fragmentation peak deriving from mechanism similar to those observed in **1**. The pseudomolecular and the sodium and potassium clustered ions appeared at *m/z* 240, 262 and 278, respectively, in the (+)ESIMS spectrum. When compared to that of **1**, the IR spectra of **3** and **4** showed the significant presence of bands due to hydroxy groups, while the UV spectra were quite similar. However, the most significant differences were observed when the ^{1}H and ^{13}C NMR spectra of **3** and **4** were compared to those of **1**. The ^{1}H NMR spectrum of **3** (Table 3) showed as the only difference the significant presence of an AB system appearing as to doublets ($J = 18.7$ Hz) at δ 4.87 and 4.84 due to the new OCH_2-7 group of the side chain attached at C-3 of the 2-pyridone ring and the absence of the singlet of the oxiran ring (H-2 in **1**). The latter was always absent in the ^{1}H NMR spectrum of **4** (Table 3) which differed from that of **1** for the only presence of the singlet at δ 5.80 attributed to the proton of the same carbon (C-7) which in this derivative is a secondary hydroxylated (Pretsch et al., 1989). The ^{13}C NMR spectra of **3** and **4** (Table 4) differed from that of **1** for the absence of the carbons (C-2 and C-3) of the oxiran ring and conjugated carbonyl group (C-8) of the pyridione ring. In fact, the signal of the first carbon (C-7) of the side chain appeared at δ 76.0 and 73.1 for an oxygenated methylene and methine group, respectively in **3** and **4**, while C-3 and C-4 of the 2-pyridone ring showed a significant change of the chemical shift. The latter appeared both as singlets at the typical chemical shift values of δ 126.6 and 159.1 and 102.0 and 165.0 (C-3 and C-4), in **3** and **4**, respectively (Breitmaier and Voelter, 1987).

Table 4
^{13}C NMR data of sapinopyridione derivatives (**2**–**4**)[a]

C	**2**	**3**	**4**
	δC[b]	δC[b]	δC[b]
2	72.8 (*d*)	161.1 (*s*)	167.4 (*s*)
3	59.7 (*s*)	126.6 (*s*)	102.0 (*s*)
4	167.8 (*s*)	159.1 (*s*)	165.0 (*s*)
5	–	99.8 (*d*)	101.0 (*d*)
6	153.8 (*s*)	140.7 (*s*)	145.9 (*s*)
7	106.6 (*d*)	76.0 (*t*)	73.1 (*d*)
8	183.9 (*s*)	214.8 (*s*)	214.9 (*s*)
9	71.2 (*d*)	43.9 (*d*)	42.3 (*d*)
10	24.4 (*d*)	25.9 (*t*)	25.7 (*t*)
11	24.5 (*t*)	11.5 (*q*)	11.6 (*q*)
12	11.2 (*q*)	15.4 (*q*)	17.6 (*q*)
13	13.9 (*q*)	19.0 (*q*)	19.0 (*q*)
14	20.7 (*q*)	–	–

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

The phytotoxic and antimycotic activity of three sapinopyridione derivatives (**2**–**4**) was evaluated in comparison to sapinopyridione (**1**).

Biological results were obtained in terms of phytotoxicity of **1** and **2**–**4** against three cypress species (Table 5). The greatest phytotoxic activity was recorded on *C. macrocarpa* and *C. sempervirens*. Dieback occurred on all twigs that absorbed a solution containing compound **1** at 100 μg ml^{-1}. Compound **1** was moderately toxic to *C. arizonica*.

Derivatives **2**, **3** and **4** were practically ineffective. The degree of phytotoxicity elicited by each compound allowed us to ascertain some structure–activity relationships as well

Table 5
Symptoms caused by sapinopyridione (**1**) and its derivatives (**2**–**4**) on test plants[a]

Compound	Concentration ($\mu g\ ml^{-1}$)	Cypress species		
		Cupressus arizonica	*Cupressus macrocarpa*	*Cupressus sempervirens*
1	50	None	Yellowing	Yellowing
	100	Yellowing	Dieback	Dieback
2	50	None	None	None
	100	None	Yellowing	Yellowing
3	50	None	None	None
	100	None	Yellowing	Yellowing
4	50	None	None	None
	100	None	None	Yellowing

[a] Symptoms developed on severed twigs within 14 days.

as to determine the structural features of each molecule responsible for the bioactivity. Results of phytotoxicity tests on *Cupressus* species were dissimilar and it might be possible to use sapinofuranones and sapinopyridione for rapid screening of conifer sensitivity/tolerance for relative pathogenicity of *S. sapinea*.

The antimycotic activity of sapinopyridione (**1**) and its derivatives (**2**–**4**) was assayed on three fungal species belonging to the genus *Seiridium*, which are dextructive pathogens of cypress (Table 6). The statistical analysis of the data allowed to compare the sensitivity of each fungal species to the toxin and its derivatives. *S. unicorne* proved to be more sensitive to **1** whereas *S. cardinale* was quite tolerant. Derivative **2** was ineffective against *S. unicorne* and moderately toxic to *S. cardinale* and *S. cupressi*. Derivative **3** elicited no inhibition at all concentrations tested against *S. cupressi* and only at low concentration vs. *S. unicorne*. A 10-fold concentration of derivative **3** caused a moderate reduction of growth of both *S. cardinale* and *S. unicorne*. Finally, derivative **4** was no effective vs. *S. cupressi* or weakly active vs. *S. cardinale* and *S. unicorne* when assayed at low concentration. When it was assayed at a concentration 10-fold greather, a remarkable increase of growth inhibition of *S. unicorne* was observed. *S. cardinale* and *S. cupressi* were less affected. Among the three *Seiridium* species, *S. unicorne* proved to be the most sensitive. The sensitivity of *S. unicorne* to all toxic compounds tested may be explained in terms of its pathogenicity and virulence characteristics which are weaker than those elicited by *S. cupressi* and *S. cardinale* on cypress trees.

Sapipyridione contains a 2,4-pyridione and an epoxy ring. Pyrones, related to pyridiones, and epoxy group are structural features which play an important role in the biological activity of some classes of natural occurring compounds as some substituted pyrones (Evidente et al., 2003; Altomare et al., 2004) sequiterpenes eremophilanes (Capasso et al., 1984), trichothecenes, verrucarins and cytochalasins (Cole and Cox, 1981; Vurro et al., 1997; Andolfi et al., 2005) and sphaeropsidones (Evidente et al., 1998). Therefore, the marked reduction or the total loss of both phytotoxic and antifungal activities of two isomeric 4-hydroxy-6-methyl-2-pyridones (**3** and **4**) in respect to those of **1** did not surprise showing these two latter the lacking of the epoxy groups. In fact, in both derivatives **3** and **4** the epoxy group is open and converted into an ether and a secondary hydroxy group, respectively. Furthermore, in both derivatives (**3** and **4**) the pyridione ring is converted into a 2-pyridone ring, which could isomerise to the 2-hydroxypyridine tautomer and, therefore, further contribute to determine the observed loss or marked decrease of activities. Finally, the reduction of the phytotoxic and antifungal activities showed by reduced derivative **2**, in respect to those of sapipyridione, also showed a role of the carbonyl group present in the side chain attached at C-3 of the pyridione ring to impart activity.

The antimycotic activity of sapinofuranones and sapinopyridione may help the saprophytic survival of *S. sapinea* in its natural habitat, or it can preserve the fungus from the frequent attacks by other colonising microorganisms, pathogens or saprobes. Therefore, the production of sapinofuranones and sapinopyridione during the infection process of *S. sapinea* in the bark or shoots may prevent as an antagonistic reaction, the concomitant invasion of the same host tissues by pathogens as the three *Seiridium* species. In addition, molecules that act as major determinants

Table 6
Sensitivity to sapinopyridione (**1**) and its derivatives (**2**–**4**) on three plant pathogenic fungi[a]

Compound	Concentration ($\mu g\ ml^{-1}$)	Fungal species			LSD ($P \leqslant 0.05$)
		Seiridium cardinale	*Seiridium cupressi*	*Seiridium unicorne*	
1	10	12.0 (20.27)	13.3 (21.39)	15.6 (23.26)	(5.4)
	100	17.3 (24.58)	35.0 (36.27)	40.0 (39.23)	(12.7)
2	10	8.0 (16.43)	8.3 (16.74)	n.i.	(3.5)
	100	14.7 (22.55)	16.7 (24.12)	n.i.	(4.7)
3	10	13.3 (21.39)	5.0 (12.92)	n.i.	(5.8)
	100	16.0 (23.58)	n.i.	15.6 (23.26)	(7.8)
4	10	5.3 (13.31)	n.i.	6.7 (15.00)	(4.2)
	100	14.7 (22.55)	16.6 (24.04)	42.2 (40.51)	(12.3)

[a] The measured values (linear growth inhibition) are the mean of 10 replicates. Angular transformation of percentage data and LSD (least significative difference) are shown in parentheses. n.i.: no growth inhibition.

of pathogenicity and virulence in plant pathogenic fungi are candidate targets when specific antifungal compounds are designed because their roles are essential during the infection process and disease development. There is a great potential reservoir of biologically active substances, which are particularly suitable for interactions with biological systems.

3. Experimental

3.1. General

Mps are uncorr. and were determined on a Gallekamp Melting Point Apparatus. The optical rotations were measured in $CHCl_3$ (unless otherwise noted) on a JASCO P1010 digital polarimeter, and the CD spectra were recorded in MeOH solution on a JASCO J-710 spectropolarimeter. IR spectra were determined as neat substance on a Perkin–Elmer Spectrum ONE FT-IR spectrometer. UV spectra were recorded in MeOH solution (unlesss otherwise noted) on a Lambda 25 UV-Visible spectrophotometer. ^1H and ^{13}C NMR spectra were recorded at 500 and 400 and at 125, 100 and 75 MHz, respectively, in $CDCl_3$, on Bruker spectrometers. The same solvent was used as internal standard. DEPT, COSY-45, TOCSY, HSQC, 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. Electrospray (ESI) MS were recorded on a Perkin–Elmer API 100 LC-MS; a probed voltage of 5300 V and a declustering potential of 50 V were used. 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 MeOH, followed by heating at 110 °C for 10 min. Column chromatography was carried out on silica gel (Merck, Kieselgel 60, 0.063–0.20 mm). Solvent systems: (A) $CHCl_3$–*iso*-PrOH (19:1), (B) EtOAc–*n*-hexane (1.5:1); (C) $CHCl_3$–*iso*-PrOH (9:1); (D) MeCN–H_2O (1:1).

3.2. Fungal strains, culture media and growth conditions

S. sapinea strains isolated from southern Italy (Cerignola, Apulia region) were obtained by direct isolation from the bark and shoots of *Cupressus macrocarpa* Hartw. (strain D-50) and *C. sempervirens* L. (strains D-54 and D-55). Four plants of *C. macrocarpa* and six plants of *C. sempervirens* showing symptoms of cankers, shoot blight and leaf necrosis were chosen to harvest samples and to attempt the isolation. Ten samples collected from bark and shoots colonised by the fungus were assessed for each cypress species. Dark-coloured fungal colonies, obtained after primary isolations, were transferred on water-agar (WA) plates supplemented with cypress needles to induce sporulation and with penicillin (50 μg ml^{-1}) and streptomycin (50 μg ml^{-1}) to prevent bacterial contamination. Identification of *S. sapinea* strains was based on conidial and pycnidial morphology (Sutton, 1980). Single conidial cultures were generated by spreading a suspension of conidia, released from excised picnidia, onto the surface of malt-agar (MA) or potato-sucrose-agar (PSA) in Petri dishes and incubated at 25 °C, in the dark or under mixed white and near-UV light, set to provide 12 h light/dark cycles. All the single conidial culture were randomly transferred to MA slants and stored at 4 °C. The fungal strains were examined and they were designated as belonging to an A morphotype, based on cultural and conidial characteristics, growth rate, growth requirements, as well a pathogenicity and host range (Frisullo et al., 1997). Growth of each strain, production of culture filtrates and extraction of secondary metabolites were managed as previously reported in detail (Evidente et al., 1999).

3.3. Extraction and purification of sapinopyridione (*1*)

The culture filtrates (4.1 l, pH 6.9) of *S. sapinea* strain D-55 was acidified to pH 4 with 1 N HCl and extracted with EtOAc (4 × 1.5 l). The combined organic extracts were dried (Na_2SO_4) and evaporated under reduced pressure to give a red-brown oily residue (4.0 g). Its TLC analysis on silica gel (eluent A) showed the presence of sapinofuranones A and B (R_f 0.44) (Evidente et al., 1999) and that of some metabolites with higher polarity. The oily mixture was chromatographed by column chromatography (eluent A) to afford 13 groups of homogeneous fractions, of which residue (226 mg) of fraction 5 contained crude sapinofuranone A and B as previously reported (Evidente et al., 1999). The residue (139 mg) of fraction 8 was chromatographed by silica gel column but eluting with solvent system B, and afforded nine groups of homogeneous fractions. The residue of fraction 3 (42 mg) was crystallised as white needles (27 mg) from a CH_2Cl_2–*n*-hexane mixture. The crystalline metabolite showed an R_f-values at 0.29 and 0.40 and 0.50 (by TLC on silica gel, eluent A and B, and on reverse phase, eluent D, respectively) and it was named sapinopyridione (**1**). The subsequent purification by prep. TLC on silica gel (eluent A) of both residues (16 and 9.7 mg) of the fractions 4 and 5 eluted from the second column, yielded further amount of pure sapinopyridione (16 mg) for a total of 43 mg (10.5 mgl^{-1}).

When the same purification procedure was applied to the oily organic crude extract (942 and 458 mg, respectively) of culture filtrates (2.4 l each) obtained from the strains D-50 (pH 6.0) and D-54 (pH 6.3), **1** was obtained at lower concentrations (5.4 and 1.1 mgl^{-1}, respectively) in comparison to the strain D-55. All the results were the means of two experiments with 26 replicates for D-50, two experiments with 24 replicates for D-54 and four experiments with 23 replicates for D-55.

3.4. Sapinopyridione (*1*)

Mp 133–135; $[\alpha]_D^{25}$ −11.3 (*c* 0.16, EtOH); CD [θ] (nm): $+1.12\times10^3$ (345), -4.78×10^2 (270), $+3.75\times10$ (222); (*c* 3.9×10^{-4} M); IR ν_{max} cm^{-1} 3258, 3189, 1722, 1668, 1629, 1251; UV (MeCN) λ_{max} (log ε) nm: 320 (3.61); ^{1}H and ^{13}C NMR: Table 1; HR EIMS (rel. int) *m/z*: 238 $[MH]^+$(1), 237.1012 $[M]^+$ (2.2) ($C_{12}H_{15}NO_4$, calc. 237.1001), 223 $[MH-Me]^+$ (0.4), 222 $[M-Me]^+$ (0.7), 209 $[M-C_2H_2]^+$ (76), 195 $[MH-Me-CO]^+$ (0.3), 194 $[M-Me-CO]^+$ (1), 181 $[MH-C_4H_9]^+$ (2), 180 $[M-C_4H_9]^+$ (3), 153 $[MH-C_4H_9-CO]^+$ (13), 152 $[M-C_4H_9-CO]^+$ (63), 126 $[MH-C_4H_9-CO-HCN]^+$ (22), 125 $[M-C_4H_9-CO-HCN]^+$ (50), 97 $[MH-C_4H_9-CO-HCN-HCO]^+$ (17), 96 $[MH-C_4H_9-CO-HCN-HCO]^+$ (19), 57 $[C_4H_9]^+$ (100). ESIMS (+) *m/z*: 238 $[M+H]^+$, 260 $[M+Na]^+$, 276 $[M+K]^+$.

3.5. 9,*O*-Dihydrosapinopyridione (*2*)

Sapinopyridione (3.7 mg) dissolved in MeOH (3.6 ml) was stoichiometrically reduced with $NaBH_4$ (0.91 mg) at 0 °C under stirring. After 1 min the reaction was stopped by addition of ice-water neutralisation with 0.1 M HCl. The MeOH was evaporated under a N_2 stream and the resulting mixture extracted with $CHCl_3$ (4×5 ml). The organic extracts were dried (Na_2SO_4), and evaporated under red. pres. to yield a crude mixture. The latter was purified by prep. TLC (eluent A) to give the 9,*O*-dihydro-derivative of **1** (**2**) as a homogeneous amorphous solid (R_f 0.15, 1.5 mg): $[\alpha]_D^{25}$ −34.6 (*c* 0.23, MeOH); CD [θ] (nm): -1.42×10^3 (340), $+1.50\times10^3$ (304), -1.41×10^3 (254), 0.65×10^3 (216) (*c* 2.17×10^{-4} M); IR ν_{max} cm^{-1} 3443, 3254, 1717, 1646, 1623, 1252; UV λ_{max} (log ε) nm: 313 (3.25); ^{1}H and ^{13}C NMR: Tables 3 and 4; HR EIMS *m/z* (rel. int.): 239.2667 $[M]^+$ (4) ($C_{12}H_{17}NO_4$ calc. 239.2677), 211 $[M-CO]^+$ (3), 193 $[M-CO-H_2O]^+$ (16), 166 $[M-CO-H_2O-HCN]^+$ (24), 152 $[M-HC(OH)CH(CH_3)CH_2CH_3]^+$ (14), 134 $[M-HC(OH)CH(CH_3)CH_2CH_3-H_2O]^+$ (50), 148 $[M-CO-H_2O-HCN-H_2O]^+$ (100),123 $[M-HCOHC(OH)CH(CH_3)CH_2CH_3]^+$ (46), ESIMS (+) *m/z*: 262 $[M+Na]^+$, 240 $[M+H]^+$, 222 $[M-H_2O]^+$; ESIMS (−) *m/z*: 238 $[M-H]^-$.

3.6. Isomeric 4-hydroxy-6-methyl-2-pyridones (*3-4*)

Sapinopyridione (**1**) (16.4 mg) dissolved in MeOH (2 ml) was added to a pre-saturated suspension of PtO_2 in MeOH (2 ml). The hydrogenation was carried out at room temperature, under atmospheric pressure with continuous stirring. After 1 h, the reaction was stopped by filtration and the clear solution evaporated under a N_2 stream. The residue was analysed by silica gel TLC (eluent C) and the conversion of **1** into two compounds with higher polarity, having R_f-values at 0.34 and 0.11 (**3** and **4**, respectively) was observed. Therefore, the reaction mixture was purified by preparative TLC, using the same conditions, affording the two structural isomeric 4-hydroxy-6-methyl–2-pyridone derivatives **3** and **4**, both as amorphous solids resistant to crystallisation (4.1 and 5.1 mg, respectively). Derivative **3** had: $[\alpha]_D^{25}$ −3.5 (*c* 0.26); IR ν_{max} cm^{-1} 3340, 1714, 1632, 1209 UV λ_{max} (log ε) nm: 280 (3.99); ^{1}H and ^{13}C NMR: Tables 3 and 4; HR EIMS (rel. int) *m/z*: 239.2688 $[M]^+$(26) ($C_{12}H_{17}NO_4$ calc. 239.2677), 224 $[M-Me]^+$ (1), 211 $[M-CO]^+$ (3), 210 $[M-HCO]^+$ (6), 196 $[M-Me-CO]^+$ (2), (78), 182 $[M-C_4H_9]^+$ (7), 155 $[MH-C_4H_9-CO]^+$ (2), 154 $[M-C_4H_9-CO]^+$ (100), 126 $[M-C_4H_9-CO-HCN]^+$ (29), 98 $[MH-C_4H_9-CO-HCN-HCO]^+$ (8), 97 $[MH-C_4H_9-CO-HCN-HCO]^+$ (9), 57 $[C_4H_9]^+$ (30). ESIMS (+) *m/z*: 240 $[M+H]^+$, 262 $[M+Na]^+$, 278 $[M+K]^+$. Derivative **4** showed: $[\alpha]_D^{25}$ −40.5 (*c* 0.18); IR ν_{max} cm^{-1} 3270, 3134, 1714, 1620, 1580, 1280; UV λ_{max} (log ε) nm: 280 (3.74); ^{1}H and ^{13}C NMR: Tables 3 and 4; HR EIMS (rel. int) *m/z*: 239.2665 $[M]^+$(2) ($C_{12}H_{17}NO_4$ calc. 239.2677), 224 $[M-Me]^+$ (1), 211 $[M-CO]^+$ (1), 210 $[M-HCO]^+$ (2.5), 196 $[M-Me-CO]^+$ (1), 182 $[M-C_4H_9]^+$ (6), 155 $[MH-C_4H_9-CO]^+$ (11), 154 $[M-C_4H_9-CO]^+$ (100), 126 $[M-C_4H_9-CO-HCN]^+$ (11), 97 $[M-C_4H_9-CO-HCN-HCO]^+$ (6), 57 $[C_4H_9]^+$ (8). ESIMS (+) *m/z*: 240 $[M+H]^+$, 262 $[M+Na]^+$, 278 $[M+K]^+$.

3.7. Phytotoxin bioassay

Culture filtrates, their chromatographic fractions and pure substances were assayed for phytotoxicity on three species of host plants (*C. sempervirens* var. *pyramidalis*, *C. macrocarpa* Hart. var. *lambertiana* and *C. arizonica* Gr.) as previously reported (Evidente et al., 1999). Sapinopyridione and its derivatives were tested at 50 and 100 μg ml^{-1}. The cuttings were left to absorb 3 ml toxic solution completely, which usually took 36 h, and were then transferred to distilled water. Toxicity symptoms were recorded after 48 h absorption period and until the 21st day. Throughout the assay, the leaves were kept in a growth chamber at relatively low temperature (23 °C), relative humidity (60%), and illumination (150 μE m^{-2} s^{-1}). Distilled water was used as control.

3.8. Antimycotic activity

The assays were carried out with *Seiridium cardinale*, *S. cupressi*, and *S. unicorne* three causal agents of various forms of canker disease on cypress, as test microorganisms. Two toxic solutions at 10 and 100 μg ml^{-1} were prepared. The test was carried out by growing each fungal species in Petri dishes containing 20 ml of potato-dextrose-agar (PDA) amended with concentrations of 10 or 100 μg ml^{-1} of each toxic substance (sapinopyridione and three key derivatives of **1**). The plates (5 per fungal species) were seeded with 2 small pieces of a 10-day-old colony mat and incubated at 25 °C for 15 days. The isolates were tested at least twice. The antifungal effect of toxins and their derivatives 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. For statistical analysis, data were expressed as angular transformations of percentage values and LSD was calculated.

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