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Ophiobolin E and 8-*epi*-ophiobolin J produced by *Drechslera gigantea*, a potential mycoherbicide of weedy grasses

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Abstract

Drechslera gigantea, a fungal pathogen isolated from large crabgrass (*Digitaria sanguinalis*) and proposed as a potential mycoherbicide of grass weeds, produces phytotoxic metabolites in liquid and solid cultures. Ophiobolin A and three minor ophiobolins i.e., 6-*epi*-ophiobolin A, 3-anhydro-6-*epi*-ophiobolin A and ophiobolin I were obtained from the liquid culture broths. Interestingly and unexpectedly, ophiobolins also appeared in cultures of this fungus and they were isolated together with the known ophiobolins B and J, and designed as ophiobolin E and 8-*epi*-ophiobolin J. They were characterized using essentially spectroscopic methods. It is noteworthy that *D. gigantea* produces such a plethora of bioactive organic substances. Some structure-activity relationship results are also discussed in this report.

Keywords: *Drechslera gigantea*; *Digitaria sanguinalis*; Ophiobolins; Large crabgrass; Phytotoxins; Ophiobolins E; 8-*epi*-Ophiobolin J

1. Introduction

Drechslera gigantea Heald & Wolf is a cosmopolitan fungal pathogen found throughout North and South America, Japan, and other regions (Sivanesan, 1992). The extensive studies carried out over the past 5 years have shown that this fungus is effective for management of grass weeds under field conditions, alone and in combination with two other grass pathogens, *Exserohilum longirostratum* and *E. rostratum* (Chandramohan and Charudattan, 2001; Chandramohan et al., 2002). *D. gigantea* causes a zonate eye-spot disease (Farr et al., 1989; IS-MPMInet, 2005) and under severe levels of disease, the leaf spots may coalesce, causing leaf lesions and leaf blight. Infected leaves may be killed.

The genus *Drechslera* is well known and widely studied because many species belonging to it are responsible for severe diseases of cereal crops (Tatum, 1972; Padmanabhan, 1973; Strobel et al., 1988). Many of these species have also been studied for the production of phytotoxic secondary metabolites, which are often involved in the infection processes. Some phytotoxins isolated from pathogens of grass weeds have been proposed as potential natural herbicides (Kastanias and Chrysayi-Tokousbalides, 2000; Kenfield et al., 1989a,b; Evidente et al., 2005). Recently, with the purpose of finding new, natural and potential herbicides, the main phytotoxic metabolite produced by *D. gigantea*, identified as ophiobolin A, was isolated from liquid fungal culture together with three minor metabolites, i.e., 6-*epi*-ophiobolin, A 3-anhydro-6-*epi*-ophiobolin A and ophiobolin I. The results of the bioassays also revealed some structural features important for the phytotoxicity (Evidente et al., 2006). The structure of ophiobolin A

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was also confirmed by direct X-ray analysis to better understand the role of the stereochemistry of its carbotricyclic ring system in the affinity with the receptors and the changes in the stereochemistry consequent to the modifications of the functional groups (Andolfi et al., 2006). Considering the interesting structure-activity results obtained, the same organic extract that yielded ophiobolin A was reinvestigated in order to identify further minor metabolites. Furthermore, the fungus was also grown on a solid medium, and the extract obtained by organic solvent extraction was analysed with the aim of finding new phytotoxic metabolites.

This paper describes the isolation, structural elucidation and initial biological characterisation of new ophiobolins produced in liquid and solid cultures by *D. gigantea*, named ophiobolin E and 8-*epi*-ophiobolin J (**6** and **8**, respectively, Fig. 1). Their structures, as well as those of the two well-known ophiobolins B and J isolated together with **6** and **8** from the fungal solid culture, were determined by spectroscopic methods (essentially NMR and MS techniques).

2. Results and discussion

The culture filtrate (2.7 l) of *D. gigantea* showing high phytotoxicity was extracted and purified as reported previously (Evidente et al., 2006), yielding ophiobolin A (**1**, Fig. 1) as the main metabolite together with 6-*epi*-, 3-anhydro-6-*epi*-ophiobolin A and ophiobolin I (**2**, **3** and **4**, Fig. 1) as minor metabolites. The residues of the ophiobolin A mother liquor crystallization were combined and purified as described in the Experimental section yielding a further ophiobolin (**6**, Fig. 1, 0.48 mg l^{-1}) as a homogeneous compound. The latter has a molecular formula of $C_{25}H_{34}O_3$ as deduced from HR ESIMS spectrum consistent with 9 of unsaturations. Compared to ophiobolin A, it showed the significant absence of one oxygen atom and the increase of one unsaturation. The preliminary ^1H and ^{13}C NMR investigations showed that it had noteworthy differences with respect to the spectra of ophiobolins (Evidente et al., 2006). However, the typical systems of the α,β-unsaturated aldehydic group of the octacyclic B ring substantially appeared unaltered (Breitmaier and Voelter, 1987; Pretsch et al., 2000) as confirmed by the analysis of the COSY and HSQC spectra (Berger and Braun, 2004). Some significant differences seemed present in both the pentacyclic rings A and C. In fact, the ketone group on C-5 and the typical AB system due to the H_2C-4 present in **1** were absent in **6**, with the consequent increase of the multiplicity and complexity of the region of methylene protons of the ^1H NMR spectrum (Table 1) and due to both H_2C-4 and H_2C-5 resonating between δ 2.45 and 1.50 and 2.05 and 1.69, respectively. Furthermore, the broad doublet ($J = 7.0$ Hz) of H-6 appeared significantly upfield shifted ($\Delta\delta$ 0.36) δ 2.85 (Pretsch et al., 2000). However, in ring A the presence of tertiary hydroxylated quaternary carbon C-3 and the corresponding geminal methyl group

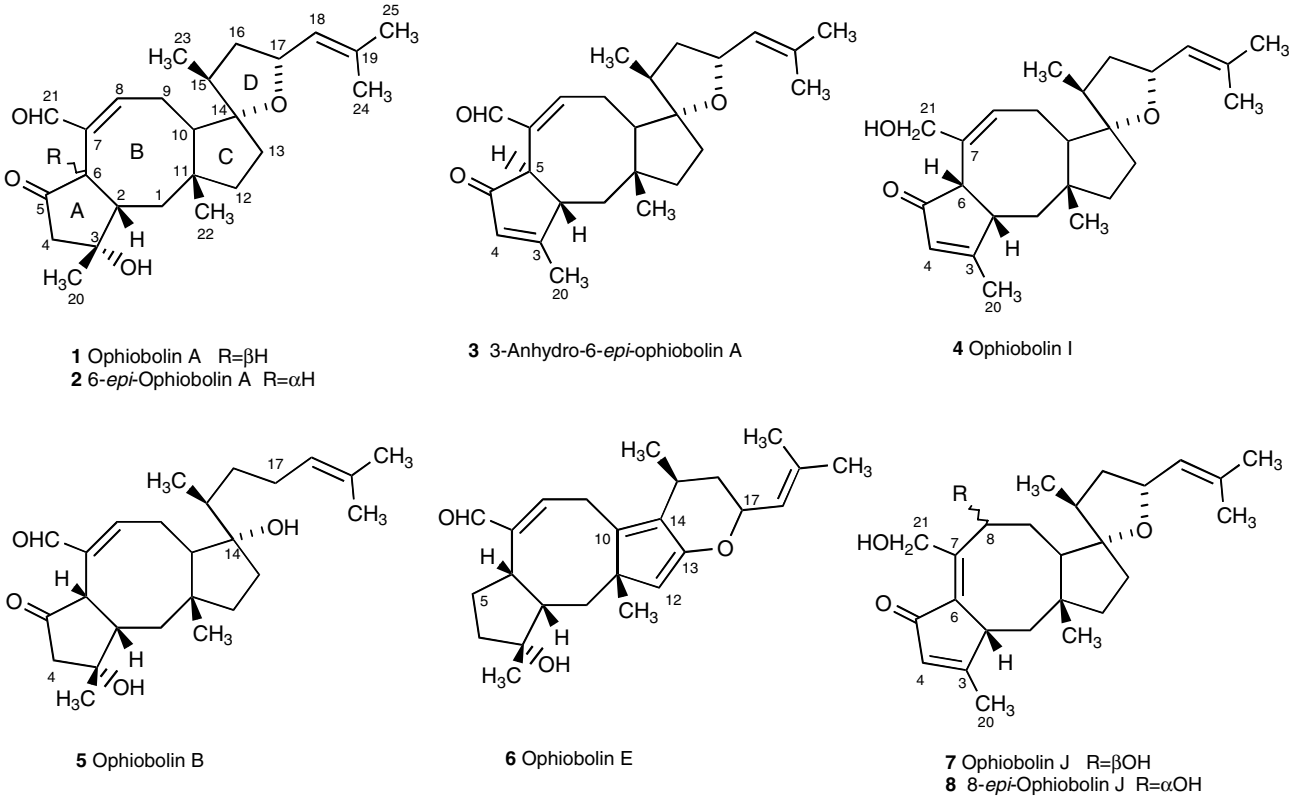

Fig. 1. Structures of ophiobolins A (**1**), B (**5**), E (**6**), I (**4**), J (**7**), 6-*epi*-ophiobolin A (**2**), 3-anhydro-6-*epi*-ophiobolin A (**3**) and 8-*epi*-ohpiobolin J (**8**).

Table 1
^{1}H and ^{13}C NMR data of ophiobolin E and 8-*epi*-ophiobolin J (**6** and **8**)[a]

C	**6**				**8**			
	δ[b]	δH	*J* (Hz)	HMBC	δ[b]	δH	*J* (Hz)	HMBC
1	30.4 t	1.50 *m* 1.17 *m*		2.85, 2.37, 0.89	49.7 t	1.96 *dd* 1.10 *m*	11.8, 1.4	3.16, 1.57, 1.34
2	45.7 d	2.37 bt	1.9	1.17, 0.89	45.8 d	3.16 *d*	11.8	2.08, 1.96, 1.10
3	78.9 s			2.85, 1.50, 1.34, 1.17	175.7 s			6.03, 3.16, 2.08, 1.96
4	53.2 t	2.45 *d* 1.50 *m*	13.1	1.69, 1.50, 1.34	131.9 d	6.03 *s*		3.16, 2.08
5	25.3 t	2.05 *m* 1.69 *m*	1.3		198.0 s			6.03, 3.16, 2.08
6	46.3 d	2.85 *br d*	7.0	9.49, 2.45, 1.50	138.6 s			6.03, 3.16, 2.08
7	141.5 s			9.49, 2.94, 2.85, 2.48, 2.45	149.0 s			1.89
8	152.7 d	7.13 *dd*	5.3, 2.0	2.94, 2.85, 2.48, 1.50, 0.89	72.1 d	4.70 *dd*	10.0, 9.9	1.89
9	26.0 t	2.94 *d* 2.48 *dd*	19.7 19.7, 5.3	7.13	34.3 t	1.89 *m* (2H)		1.89
10	158.8 s			2.20	51.1 s	1.89 *m*		1.96, 1.89, 1.68, 1.51
11	58.0 s			7.13, 2.94, 2.85, 2.48	43.6 s			1.96, 1.89, 1.68, 1.57, 1.34, 1.51, 1.10
12	124.5 d	5.08 *br t*	5.4		30.3 t	1.68 *m* 1.51 *m*		2.20, 1.57, 1.34
13	159.6 s			5.84, 3.85, 0.93	42.4 t	1.57 *m* 1.34 *m*		
14	133.0 s				95.8 s			2.20, 1.89, 1.79, 1.68, 1.51, 1.34, 1.02
15	33.0 d	2.20 *m*		1.50	36.0 d	2.20 *dq*	13.7, 6.9	1.79, 1.68, 1.02
16	35.5 t	1.69 *m* 1.50 *m*		0.93	42.0 t	1.79 *m* 1.68 *m*		2.20, 1.02
17	85.7 d	3.85 *br d*	2.9	5.84	71.9 d	4.54 *dd*	15.7, 7.2	2.20, 1.79
18	125.8 d	5.84 *br d*	2.9	1.67, 1.59	127.0 d	5.16 *br d*	7.2	1.79, 1.69, 1.64
19	134.0 s			1.67, 1.59	134.5 s			4.54, 1.69, 1.64
20	29.2 q	1.34 *s*		1.50, 1.17	17.4 q	2.08 *s*		6.03
21	193.4 *d*	9.49 *s*		7.13, 2.94	56.7 t	4.74 *br s*		
22	14.1 q	0.89 *s*			22.7 q	1.10 *s*		1.96, 1.89, 1.57, 1.34, 1.10
23	22.7 q	0.93 *d*	7.0		16.3 q	1.02 *d*	6.9	2.20, 1.79, 1.68
24[c]	18.0 q	1.59 *s*			18.1 q	1.64 *s*		5.16, 1.79
25[c]	25.6 q	1.67 *s*			25.9 q	1.69 *s*		5.16, 1.79, 1.64

The chemical shifts are in δ values (ppm) from TMS.
[a] 2D ^{1}H,^{1}H (COSY) and 2D ^{13}C,^{1}H (HSQC) NMR experiments delineated the correlations of all protons and the corresponding carbons.
[b] Multiplicities determined by DEPT spectrum.
[c] These assignments can be exchanged (they were made by comparison with analogues Li et al., 1995).

appeared evident, as their corresponding signals were observed in the ^{13}C NMR spectrum as a singlet and a quartet at typical chemical shift values of 78.9 and 29.2 (Breitmaier and Voelter, 1987). The methyl group on the other hand appeared as a singlet in the ^{1}H NMR spectrum at the expected chemical shift value of δ 1.34 (Pretsch et al., 2000). These structural features are in agreement with the signal recorded in the IR spectrum for α,β-unsaturated carbonyl and hydroxy groups (Nakanishi and Solomon, 1977), as well as with the typical maximum absorption recorded in the UV spectrum at 233 nm (Scott, 1964). Considering the lack of the ketone group at C-5 and the presence of four rings and the double bond of the isopentenyl side [C(17)-C(25)], the remaining two unsaturations of **6** should be located in the ring C. In fact, the ^{1}H spectrum of **6** showed a broad triplet ($J = 5.4$ Hz) typical of an olefinic proton (Pretsch et al., 2000) at δ 5.08 and, compared to the spectrum of ophiobolin A, the absence of the signals of H-10 as well as those of the two methylene groups H_2C-12 and H_2C-13. The comparison of the corresponding ^{13}C NMR spectra showed the absence in **6** of the significant oxygenated quaternary carbon of C-14, and the methine and the methylene carbons of C-10, C-12 and C-13, while four olefinic carbons were present at the typical chemical shift values expected for a suitable substituted 1,3-cyclopentadienylic ring (Breitmaier and Voelter, 1987). Of these, the secondary carbon at δ 124.5 was attributed to C-12 while the three remaining quaternary carbons present at δ 159.6, 158.8 and 133.0 were assigned to C-13, C-10 and C-14, respectively, on the basis of the couplings observed in the HMBC spectrum (Table 1) (Berger and Braun, 2004). The 1,3-dienylic nature of the ring C was in agreement with the typical bands observed in both IR (Nakanishi and Solomon, 1977) and UV spectra (Scott, 1964) of **6**. Finally, the ether bridge of the D ring should be present between C-17 and C-13, considering the typical chemical shift value of δ 159.6 shown by C-13 in the ^{13}C NMR spectrum (Breitmaier and Voelter, 1987). Consequently, **6** represents the first ophiobolin in which the D ring has become a substituted dihydropyran ring, joined with the C ring through the C(13)–C(14) side bond and bearing the secondary methyl group and the 2,2-dimethylvinylidene side chain

at 4- and 2-positions in respect to the oxygen atom. The chemical shifts of the secondary methyl group (Me-CH-15) as well as those of the dimethylvinylidene tail at C-17 are very similar to those reported for the ophiobolin A (Evidente et al., 2006). Instead, the signals of H-17 and H-18, both resonating as broad doublets at δ 3.85 and 5.84 in the ^{1}H NMR spectrum (Pretsch et al., 2000), appear substantially different. Likewise, the C-17 resonated downfield shifted ($\Delta\delta$ 14.9) at δ 85.7 in the corresponding ^{13}C NMR spectrum (Breitmaier and Voelter, 1987).

On the basis of the correlations observed in the COSY and HSQC spectra, the chemical shift was attributed to all the protons and the corresponding carbons (Table 1). The structure of this ophiobolin is depicted in **6** (Fig. 1). Considering that the name ophiobolin E does not appear to be attributed to any compound (Au et al., 2000), we decided to assign this name to **6**.

The structure of ophiobolin E was supported by several ^{1}H,^{13}C long-range correlations and the effects recorded for **6** in the HMBC and NOESY spectra (Tables 1 and 2) (Berger and Braun, 2004), and by data of its HR ESI MS spectra. The latter, recorded in positive mode, in addition to the sodium cluster $[M + Na]^+$ at *m/z* 405.2416, showed the potassium $[M + K]^+$ cluster and the pseudomolecular ions at *m/z* 421and 383, respectively. When recorded in negative modality, the ESI MS spectrum showed the significant pseudomolecular ion $[M\text{-}H]^-$ at *m/z* 381.

When grown as solid culture, *D. gigantea* produced different ophiobolins. The organic extract was purified by a combination of column and preparative TLC, as described in the Experimental section, giving four ophiobolins, all isolated as amorphous solids. Three of them were identified as ophiobolins A (obtained in very low amounts with respect to the liquid culture of the same fungus), B and J (**5** and **7**, Fig. 1), by comparison of their spectroscopic properties, essentially the ^{1}H and ^{13}C NMR and MS data. Their physical and spectroscopic data were very similar to those reported in the literature (Evidente et al., 2006; Li et al., 1995; Sugawara et al., 1988). The fourth ophiobolin (**8**, Fig. 1) appeared to be a new compound closely related to ophiobolin J, as shown by the same molecular formula of $C_{25}H_{36}O_4$ deduced from its HR ESIMS and by the comparison of their IR, UV and ^{1}H and ^{13}C NMR spectra. In particular, the only significant difference observed in the ^{1}H NMR spectrum was the signal of H-8 which appears in both compounds as a double doublet at δ 4.68 and 4.70 in **7** and **8**, respectively, but differently coupled with the protons of the adjacent H_2C-9. In fact, the coupling constants measured for H-8 were 10.0 and 9.9 Hz in **8** while the same in **7** were 5.7 and 4.5 Hz respectively. On the basis of these results, the structure of 8-*epi*-ophiobolin J was assigned to **8** (Fig. 1). This is the first ophiobolin showing the epimerization of C-8.

The correlations observed in the COSY and the HSQC spectra allowed the assignment of the chemical shift to all the protons and the corresponding carbon of **8** (Table 1) which, as expected, were very similar to those of **7** (Sugawara et al., 1988).

The structure of 8-*epi*-ophiobolin J (**8**) was supported by several ^{1}H,^{13}C long-range correlations and the effects were recorded for **8** in the HMBC and NOESY spectra (Tables 1 and 2). Particularly significant was the clear NOE effect observed between H-8, having a β-position, and the Me-22 located on the same side of the molecule.

The structure of **8** was also supported by the data of its HR ESI MS spectra. The latter, recorded in positive mode, in addition to the sodium cluster $[M + Na]^+$ at *m/z* 423.2515, showed the potassium $[M + K]^+$ cluster and the pseudomolecular ions at *m/z* 439 and 401, respectively, and the ion at *m/z* 383 generated from this latter by the loss

Table 2
2D ^{1}H-NOE (NOESY) data obtained for ophiobolin E and 8-*epi*-ophiobolin J (**6** and **8**)

6		**8**	
Considered	Effects	Considered	Effects
9.49 (H-21)	7.13 (H-8), 2.85 (H-6), 1.17 (H-1′)	5.16 (H-18)	4.54 (H-17), 2.20 (H-15), 1.79 (H-16), 1.68 (H-16′)
7.13 (H-8)	9.49 (H-21), 2.94 (H-9), 2.48 (H-9′), 0.93 (Me-23)	4.74 (H_2-21)	4.70 (H-8), 3.16 (H-2)
5.84 (H-18)	3.85 (H-17), 2.20 (H-15), 1.69 (H-16), 1.50 (H-16′), 0.93 (Me-23)	4.70 (H-8)	4.74 (H_2-21), 1.89 (H_2-9), 1.10 (Me-22)
5.08 (H-12)	2.20 (H-15), 1.69 (H-16), 1.50 (H-16′)	4.54 (H-17)	5.16 (H-18), 1.79 (H-16), 1.68 (H-16′), 1.02 (Me-23)
3.85 (H-17)	5.84 (H-18), 1.50 (H-16′), 0.93 (Me-23)		
2.94 (H-9)	7.13 (H-8), 2.48 (H-9′), 2.20 (H-15), 0.93 (Me-23)	3.16 (H-2)	4.74 (H_2-21), 1.96 (H-1), 1.10 (Me-22)
2.85 (H-6)	9.49 (H-21), 2.37 (H-2), 1.50 (H-4′), 1.34 (Me-20)	2.20 (H-15)	5.16 (H-18)
2.48 (H-9′)	9.49 (H-21), 2.94 (H-9), 0.89 (Me-22)	1.96 (H-1)	3.16 (H-2)
2.45 (H-4)	1.50 (H-4′), 1.34 (Me-20), 1.17 (H-1′)	1.89 (H_2-9)	4.70 (H-8)
2.37 (H-2)	2.85 (H-6), 1.50 (H-1)	1.79 (H-16)	5.16 (H-18), 4.54 (H-17)
2.20 (H-15)	2.94 (H-9), 1.69 (H-16), 1.50 (H-16′), 0.93 (Me-23)	1.68 (H-16′)	5.16 (H-18), 4.54 (H-17)
1.69 (H-16)	5.84 (H-18), 5.08 (H-12), 2.20 (H-15)	1.10 (Me-22)	4.70 (H-8), 3.16 (H-2)
1.50 (H-16′)	5.84 (H-18), 5.08 (H-12), 3.85 (H-17), 2.20 (H-15)	1.02 (Me-23)	4.54 (H-17)
1.50 (H-4′)	2.85 (H-6), 2.45 (H-4)		
1.50 (H-1)	2.37 (H-2)		
1.34 (Me-20)	2.85 (H-6), 2.45 (H-4)		
1.17 (H-1′)	9.49 (H-21), 2.45 (H-4)		
0.93 (Me-23)	7.13 (H-8), 5.84 (H-18), 3.85 (H-17), 2.94 (H-9), 2.20 (H-15)		
0.89 (Me-22)	2.48 (H-9′)		

Table 3
Effect of ophiobolins B and J, (**5** and **7**, respectively) on various weed species tested by the leaf-puncture assay

Species	Compound[a]	
	Ophiobolin B **(5)**	Ophiobolin J **(7)**
Avena sterilis	++[b]	+
Bromus sp.	++++	++
Hordeum murinum	++++	++
Oryzopsis miliacea	+	−

Observations were made 2 days after droplet application.
[a] 0.5 mg ml^{-1}–droplets 15 μl.
[b] Diameter of necrosis on leaves: ++++ = necrosis diameter > 6 mm; ++ = necrosis between 4 and 2 mm; + = necrosis between 2 and 1 mm; − = no necrosis.

of H_2O. When recoded in negative modality, the ESI MS spectrum showed the significant pseudomolecular ion $[M-H]^-$ at *m/z* 399.

Tested at the concentration of 0.5 mg ml^{-1} on four weedy plants using the leaf-puncture assay, only ophiobolins B and J proved to be toxic (Table 3), whereas the two new ophiobolins, ophiobolin E and 8-*epi*-ophioblin J, appeared to be inactive on all the tested plant species. In particular, ophiobolin B was highly toxic to *Bromus* sp. and *Hordeum marinum* leaves, but less toxic to the other two weed species. The same range of toxicity, but at a lower level, was observed for ophiobolin J.

The modulated activity of ophiobolin B (**5**) on the different tested plants appears to be similar to that previously reported for ophiobolin A (**1**). This result was predictable because the two ophiobolins are structurally closely related. Moreover, ophiobolin J (**7**), having reduced or no activity, is related to ophiobolin I (**4**), which had proved to be inactive (Evidente et al., 2006). This activity is in agreement with the phytotoxicity previously observed for the same toxin (Sugawara et al., 1988). The different levels of phytotoxicity shown by the two ophiobolins J and I could be attributed to the different conformation that the octacyclic B ring can assume, as a consequence of the different position of the double bond, which is located between C-7 and C-8 in **4**, and between C-6 and C-7 in **7**. Probably, when present, the epimerization of the hydroxy group of C-8, observed for the first time in **8**, imparts the total loss of the activity. The noteworthy structural differences present in ophiobolin E could justify the observed inactivity on the tested plants. In fact, this latter ophiobolin showed the conversion of the cyclopentane C ring, present in all the other ophiobolins, into a 1,3-cyclopentadiene joined with the D ring, which in turn is present for the first time as a tetrasubstituted dihydropyran ring. Consequently, the configuration of the octacyclic B ring as well as that of the 2,2-dimethylvinylidene residue at C-17 should be substantially changed. Moreover, as a further difference in respect to the other ophiobolins, **6** showed the lack of the ketone group at C-5, which determines a different A ring conformation.

In conclusion, when grown in solid culture, *D. gigantea* produces ophiobolin A, but at a very low level compared to the amount recovered from the liquid culture, and some different related metabolites that show novel chemical structures and different biological activities. The availability of different compounds that are structurally related could contribute to clarify the structure-activity relationships within the ophiobolin family. Included in this family are sesterterpenoids with a common basic structure produced by different phytopathogenic fungi that are widely studied for their interesting biological activity (Au et al., 2000). This knowledge can be of great importance in understanding the site of action of the toxins, in preparing new derivatives with enhanced or modified biological activities, and for the synthesis of bioactive compounds. In view of a potential practical application of those compounds as natural and selective herbicides, this information has great value.

3. Experimental

3.1. General

Optical rotation was measured in $CHCl_3$ solution on a Jasco P-1010 digital polarimeter; IR spectra were recorded as neat on a Perkin-Elmer Spectrum One FT-IR Spectrometer and UV spectra was taken in MeCN solution on a Perkin-Elmer Lambda 25 UV/Vis spectrophotometer. ^1H and ^{13}C NMR spectra were recorded at 600, 400 and at 150, 100 and 75 MHz, respectively, in $CDCl_3$ on Bruker spectrometers. The same solvent was used as internal standard. Carbon multiplicities were determined by DEPT spectra (Berger and Braun, 2004). DEPT, COSY-45, HSQC, HMBC and NOESY experiments (Berger and Braun, 2004) were performed using Bruker microprograms. EI MS were taken at 70 eV on a Fisons Trio-2000. Electrospray MS were recorded on a Perkin-Elmer API 100 LC-MS, with a probed voltage of 5300 V and a declustering potential of 50 V. HR ESI MS spectrum was recorded on Micromass Q-TOF Micro. 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 visualized by exposure to UV light and/or by spraying first with 10% H_2SO_4 in methanol and then with 5% phosphomolybdic acid in ethanol, followed by heating at 110 °C for 10 min. CC: silica gel (Merck, Kieselgel 60, 0.040–0.063 mm). Solvent systems used were: (A) $CHCl_3$-*iso*-PrOH (24:1); (B) EtOAC-*n*-hexane (6:5); (C) $CHCl_3$-*iso*-PrOH (7:3); (D) EtOH-H_20 (3:2); (E) petrol-Me_2CO (7:3).

3.2. Fungus

D. gigantea was isolated during extensive field surveys in Florida from naturally infected large crabgrass (*Digitaria sanguinalis*) (Chandramohan and Charudattan, 2001). It was stored as PDA slants both in the Biological Control of Weeds Collection at the Plant Pathology Department,

University of Florida/IFAS, Gainesville, FL, USA (N. LCLF-1) and in the collection of the Institute of Science of Food Production, Bari, Italy (strain N. 7004).

3.3. Production, extraction and purification of *D. gigantea* ophiobolins

The fungus was grown in liquid culture as previously reported (Evidente et al., 2006). The organic extract (393.5 mg) obtained from the culture filtrate (2.7 l) was purified by silica gel column eluted with the solvent system A, obtaining 10 groups of homogeneous fractions. The residue of the third fraction (149 mg) was crystallized with ethyl acetate-*n*-hexane (1:5; v:v, three times) and gave the main metabolite (43 mg) as a white crystal. The pure metabolite was identified as ophiobolin A (**1**) (Evidente et al., 2006). The residues obtained from the mother liquors after ophiobolin A crystallization (55 mg) were combined and further purified by preparative TLC (eluent B) producing three bands. The residue of the third band (R_f 0.80) gave a homogenous oil, named ophiobolin E (**6**, 1.3 mg, 0.48 mg l^{-1}).

For the production of toxins, the fungus was also grown on a solid medium. Steamed and autoclaved wheat kernels placed in 1 l flasks were seeded using a spore suspension of the fungus, and kept at 25 °C for 4 weeks. After incubation and fungal growth, the kernels were dried and finely minced. 1kg of dried material was extracted with a MeOH–H_2O (1% NaCl) mixture (55:45, v/v), de-fatted by *n*-hexane extraction, and then extracted with CH_2Cl_2. The CH_2Cl_2 extracts were combined, dehydrated by Na_2SO_4 and evaporated under reduced pressure yielding a brown oil (781.3 mg) showing strong phytotoxic activity when assayed as described below (3.6). The latter was fractionated by CC eluted with solvent system A, yielding 10 groups of homogeneous fractions, weighing 2.4, 4.8, 46.0, 5.5, 128.5, 9.7, 27.9, 49.3, 30.0, and 474.2 mg, respectively.

The purification of the residue of the third group (46 mg) by two successive preparative TLC steps on silica gel (eluent A and B, respectively) gave a very small amount of ophiobolin A (**1**, 1 mg kg^{-1}) as an amorphous solid. The residue of the seventh fraction (27.9 mg) was purified by preparative TLC (silica gel, eluent C) producing four bands. The first of them (14.2 mg) was further purified using two successive steps by preparative TLC on reversed-phase (eluent D, R_f 0.23) and by silica gel (eluent E), respectively, yielding a white amorphous solid (R_f 0.30, 1.4 mg kg^{-1}) named 8-*epi*-ophiobolin J (**8**). The residue of the eighth fraction (49.3 mg) was purified by preparative TLC (silica gel, eluent A) producing six bands. The most polar of them (12.6 mg) was further purified by preparative TLC (silica gel, eluent E) yielding ophiobolin B (**5**) as a white amorphous solid (R_f 0.49, 1.2 mg kg^{-1}). The residue of the ninth fraction (30 mg) was purified by preparative TLC (eluent A) producing six bands. The first of them (8.9 mg kg^{-1}) appeared to be a homogeneous amorphous solid (R_f 0.23 and R_f 0.49, silica gel eluent A and E) and was identified as ophiobolin J (**7**).

3.4. Ophiobolin E (6)

Compound **6** had: $[\alpha]_D^{25} + 10.4°$ (c 0.16), IR ν_{max} 3435, 1682, 1629 cm^{-1}; UV λ_{max}nm (log ε) 233 (3.2), 220 (3.11); ^1H and ^{13}C NMR spectra: see Table 1; HR ESI-MS (+) *m/z* 421 $[M+K]^+$, 405.2412 $[M+Na]^+$ (calcd. for $C_{25}H_{34}O_3Na$, 405.2406), 383 $[M+H]^+$; ESI-MS (−) *m/z*: 381 $[M-H]^-$.

3.5. 8-epi-Ophiobolin J (8)

Compound **8** had: $[\alpha]_D^{25} + 31.1$ (c 0.1); IR ν_{max}: 3388, 1673, 1614 cm^{-1}; UV λ_{max} nm (log ε) 262 (3.64); ^1H and ^{13}C NMR spectra: see Table 1; HR ESI-MS (+) *m/z* 439 $[M+K]^+$, 423.2515 $[M+Na]^+$ (calcd. for $C_{25}H_{36}O_4Na$, 423.2511), 401 $[M+H]^+$, 383 $[M+H-H_2O]^+$; ESI-MS (−) *m/z*: 399 $[M-H]^-$.

3.6. Biological assay

The organic extract of the fungal culture filtrates as well as ophiobolins B, E, J and 8-*epi*-J were assayed for their phytotoxicity on four weedy plants, as reported in Table 3, using the methods described in details in our recent publication (Evidente et al., 2006). Pure compounds were tested at the concentration of 0.5 mg ml^{-1}. Observations were made 2 days after droplet application.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2006.07.016.

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