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Cytochalasins Z1, Z2 and Z3, three 24-oxa[14]cytochalasans produced by *Pyrenophora semeniperda*

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Abstract

Three new cytochalasans, named cytochalasins Z1, Z2 and Z3, were isolated from the wheat culture of *Pyrenophora semeniperda*, a fungus proposed to biologically control grass weeds. Other cytochalasins isolated from the same organic extract were identified as the already known cytochalasins F, T, deoxaphomin and cytochalasins B, the latter being produced in very large amounts. All three new cytochalasins were characterized as 24-oxa[14]cytochalasans by extensive use of NMR and MS techniques. Cytochalasins Z1 and Z2 proved to be structurally related to cytochalasin T, whereas cytochalasin Z3 was related to cytochalasin B. When assayed on wheat and tomato seedlings, cytochalasin Z3, in comparison to the new cytochalasins, cytochalasin B, its 21,22-dihydroderivative, cytochalasin F and deoxaphomin showed a remarkable ability to inhibit root elongation. The possibility of using these metabolites in biological control strategies is discussed.

Keywords: Grass weeds; *Pyrenophora semeniperda*; Phytotoxins; Cytochalasins; 24-Oxa[14]cytochalasans; Cytochalasins Z1, Z2 and Z3; Weed biocontrol

1. Introduction

Of all the possible causes of loss in cereal yields, weeds, such as annual grasses are one of the most important; this is due to their similarity in morphology, physiology and ecology to the crop species. A feature common to annual grasses is their prodigious seed production, which is responsible for their reproduction and diffusion, even if their viability is low. Tactics that reduce the input of seed can improve long-term control of infesting grasses. Furthermore, the increasing number of weed species which are tolerant or resistant to the use of herbicides re-emphasizes the need to develop new biological control strategies.

One such strategy could be the massive application of seed-borne pathogens as bioherbicides. Pathogens damaging the seed in the inflorescence or preventing flowering have also potential for biological control. Agents that attack the reproductive output of weeds are frequently used in biological control programmes against weeds in pastures, rangeland and natural habitats. *Pyrenophora semeniperda* (Brittlebank & Adam) Shoemaker, a seed-borne pathogen that causes several symptoms in infected plants, has been proposed as a bioherbicide (Campbell et al., 1996). *P. semeniperda* was first described in Europe in 1841, and later in Australia, New Zealand, North America and South Africa. The fungus infects seeds and leaves of over 35 genera of grasses including all the winter cereals and six dicotyledonous genera (Medd, 1992). In brome grass (*Bromus* spp.) and wheat (*Triticum aestivum* L.) it has been reported to cause death of seed primordia and subsequent abortion of seed (Neergard, 1979). The most striking symptom is the production of vegetative fungal stromata on infected seeds, which can

Abbreviations: cytochalasin Z1 (**5**): 16-methyl-10-(4-hydroxy)-phenyl-24-oxa[14]cytochalasa-6(7),13,21-triene-1,23-dione (7*Z*,13*E*,16*R*, 21*E*); cytochalasin Z2 (**6**): 12,20-dihydroxy-16-methyl-10-phenyl-24-oxa[14]5cytochalasa-6(7),13,21-triene-1,23-dione(7*Z*,13*E*,16*R*,21*E*); cytochalasin Z3 (**7**): 7,19-dihydroxy-16-methyl-10-phenyl-24-oxa[14]cytochalasa-6(12),13,21-triene-1,23-dione(7*S*,13*E*,16*R*,21*E*).

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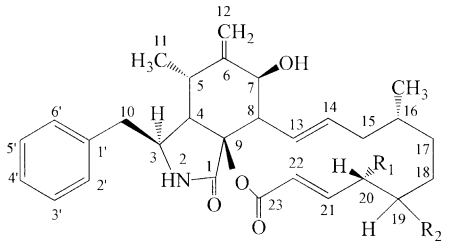
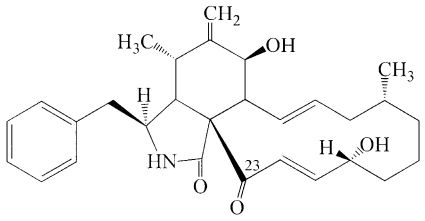
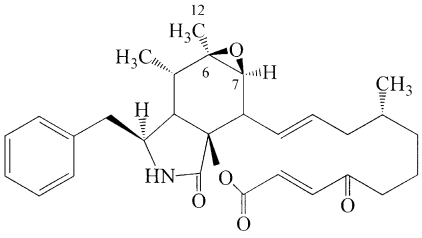


Cytochalasin B, **1** R_1=OH, R_2=H

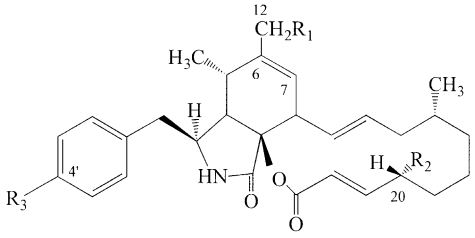
Cytochalasin Z3, **7** R_1=H, R_2=OH



Deoxaphomin, **2**



Cytochalasin F, **3**



Cytochalasin T, **4** R_1=R_3=H, R_2=OH

Cytochalasin Z1, **5** R_1=R_2=H, R_3=OH

Cytochalasin Z2, **6** R_1=R_2=OH, R_3=H

lead to a reduction in the germination capacity or a decrease in seedling vigour. The ability of *P. semeniperda* to infect seeds when applied as conidial suspension to the inflorescence of several grassy weed-species has also been demonstrated. Since some annual grasses may occur in pastures or crops used as forage, any potential bioherbicidal agent should be devoid of toxic effects on livestock. Equally, there should be no risk of introducing toxins to grains that are harvested for human consumption.

It is well known that other species of *Pyrenophora* produce toxins, some of which are potentially dangerous (Bach et al., 1979; Friis et al., 1991). Considering the interest for bioactive metabolites produced by weed pathogens to be employed either in addition or as an alternative to the use of the pathogen, it seemed interesting to investigate the production of toxins by this species of *Pyrenophora*. Preliminary in vitro experiments showed that the fungus in liquid culture produces low-molecular lipophilic phytototoxins, whose isolation, chemical and biological characterization is in progress. When grown on wheat kernels it does not produce those phytotoxins but cytochalasins, a large group of fungal metabolites having different biological activities (Cole and Cox, 1981; Natori and Yahara, 1991; Abate et al., 1997; Vurro et al., 1997; Evidente and Motta, 2001). This work reports the chemical characterization of three new cytochalasins. They were isolated together with four already known cytochalasins; cytochalasin B, produced in very large amounts, cytochalasins F and T, and deoxaphomin. Considering their inhibitory effect on root elongation, we will discuss them together with other phytotoxins produced by the same fungus, in the context of biological control strategies.

2. Results and discussion

Fungal cultures of *P. semeniperda* grown on wheat kernels were extracted with a water–methanol mixture, defatted and re-extracted with methylene chloride, then dried to give an abundant solid residue mixed with a brown oil (4.76 g kg^{-1}). It was washed with small aliquots of methanol and the residue crystallised from EtOAc-*n*-hexane gave the main metabolite. It was identified as the well known cytochalasin B (CB, **1**, 2.21 g kg^{-1}) by comparing its spectroscopic (^1H and ^{13}C NMR and MS spectra) and chromatographic behaviour with those of a standard sample (Capasso et al., 1987). The mother liquors of the cytochalasin B crystallisation were combined with the above cited methanolic washes and fractionated by silica gel column (eluent A), as described in detail in the experimental section. Nine groups of homogeneous fractions were collected and TLC monitored (eluents A and B) in comparison with several standard cytochalasins, showing the predominant presence of cytochalasin B, as well as that of other probably known and new cytochalasins. Further chromatographic purifications of some of these fractions yielded another

amount (1.43 g kg^{-1}, for a total amount of 3.64 g kg^{-1}) of cytochalasin B, deoxaphomin (DEOXA, **2**, 12 mg kg^{-1}) and cytochalasins F and T (CF, **3**, 20 mg kg^{-1} and CT, **4**, 13 mg kg^{-1}). They were identified by comparing their spectroscopic and chromatographic properties with those of standard samples of **2**, **3** and **4** (Capasso et al., 1988, 1991). Furthermore, two other homogeneous amorphous solids, obtained from the same purification process, appeared by preliminary spectroscopic investigation to be two new 24-oxa[14]cytochalasans and therefore were named cytochalasins Z1 (CZ1, **5**, 11 mg kg^{-1}) and Z2 (CZ2, **6**, 11 mg kg^{-1}).

From the crude organic extract of the same *P. semeniperda* solid culture (780 g), further amounts of crystalline cytochalasin B (2.3 g) as well as of the above cited cytochalasins were obtained, together with small amounts (1.3 mg, 1.6 mg kg^{-1}) of another homogeneous amorphous solid. The latter compound appeared also to be a new 24-oxa[14]cytochalasan through preliminary spectroscopic investigation, and was therefore named cytochalasin Z3 (CZ3, **7**).

Spectroscopic (IR, UV ^1H and ^{13}C NMR) investigation carried out on cytochalasins Z1, Z2 and Z3 (**5**, **6** and **7**) showed the presence of three structural components present in all the known cytochalasins (Cole and Cox, 1981; Natori and Yahara, 1991; Vurro et al., 1997): the highly substituted perhydroisoindol-1-one fused with the macrocyclic lactone ring and the benzyl or a *p*-monosubstituted benzyl residue at C-3.

Cytochalasin Z1 (**5**), with molecular weight of 463 as established by EI, ES and FAB mass spectra, appeared to be closely related to cytochalasin T. In fact, as already observed in **4** (Capasso et al., 1991), the ^1H NMR spectra of **5** (Table 1) differed from that of **1**

Table 1
^1H and ^{13}C NMR spectral data of cytochalasins Z1, Z2 and Z3 (**5**, **6** and **7**, respectively). The chemical shift are in δ-values (ppm) from TMS[a]

C[b]	**5**			**6**			**7**		
	δC	δH	*J* (Hz)	δC	δH	*J* (Hz)	δC	δH	*J* (Hz)
1	172.0 *s*			171.4 *s*			171.1 *s*		
3	59.6 *d*	3.06 *ddd*	10.4, 3.9, 3.6	55.9 *d*	3.31 *ddd*	9.7, 4.1, 4.0	53.5 *d*	3.30 *m*	
4	52.1 *d*	2.70 *dd*	4.0, 3.9	51.2 *d*	2.78 *dd*	4.0, 3.9	49.4 *d*	2.79 *dd*	4.0, 4.0
5	34.5 *d*	2.81 *m*		33.8 *d*	3.06 *m*		33.7 *d*	3.28 *m*	
6	140.2 *s*			143.1 *s*			148.3 *s*		
7	124.2 *d*	5.38 *br s*		127.1 *d*	5.64 *br s*		68.5 *d*	3.86 *d*	11.2
8	46.9 *d*	3.20 *br d*	10.2	45.6 *d*	3.41 *br d*	10.3	49.8 *d*	3.21 *dd*	11.2, 10.7
9	86.1 *s*			86.4 *s*			83.1 *s*		
10	43.0 *t*	2.92 *ddd*	13.4, 3.6	44.2 *t*	2.91 *dd*	13.4, 4.1	44.0 *t*	2.90 *dd*	13.4, 9.6
		2.81 *dd*	13.4, 10.4		2.88 *dd*	13.4, 9.7		2.84 *dd*	13.4, 4.5
11	14.2 *q*	1.22 *d* (3H)	6.6	43.0 *q*	1.31 *d* (3H)	7.3	14.4 *q*	1.11 *d* (3H)	6.6
12	19.9 *q*	1.77 *br s* (3H)		63.5 *t*	4.15 *br s* (2H)		114.6 *t*	5.41 *br s*	
								5.18 *br s*	
13	128.6 *d*	5.87 *ddd*	14.4, 10.2, 1.8	127.9 *d*	5.95 *ddd*	14.4, 10.3, 1.8	126.5 *d*	5.98 *br dd*	14.7, 10.7
14	134.3 *d*	5.35 *ddd*	14.4, 10.9, 3.7	143.1 *d*	5.31 *ddd*	14.4, 10.7, 3.2	138.0 *d*	5.47 *ddd*	14.7, 10.9, 3.3
15	41.1 *t*	2.12 *br d*	13.8, 1.8	41.2 *t*	2.11 *br d*	13.5, 1.8	41.3 *t*	2.16 *br d*	13.7
		1.77 *m*			1.77 *m*			1.79 *m*	
16	31.3 *d*	1.41 *m*		33.0 *d*	1.41 *m*		31.9 *d*	1.33 *m*	
17	19.9 *t*	1.60 *m*		34.6 *t*	1.40 *m*		30.7 *t*	1.64 *m*	
		0.93 *m*			0.92 *m*			0.79 *m*	
18	20.3 *t*	1.19 *m*		20.4 *t*	1.47 *m*		37.7 *t*	1.88 *m*	
		0.78 *m*			0.73 *m*			1.33 *m*	
19	34.3 *t*	1.73 *m*		34.7 *t*	1.86 *m*		71.9 *d*	3.71 *m*	
		1.35 *m*			1.67 *m*				
20	34.1 *t*	2.28 *m*		71.3 *d*	4.47 *m*		42.7 *t*	2.74 *m*	
								2.25 *ddd*	8.6, 8.4, 8.4
21	152.6 *d*	7.17 *ddd*	15.6, 9.0, 6.5	151.7 *d*	7.08 *dd*	15.7, 6.0	146.8 *d*	7.03 *ddd*	15.6, 8.4, 8.4
22	121.0 *d*	5.69 *d*	15.6	119.7 *d*	5.89 *dd*	15.7, 1.7	122.7 *d*	5.75 *d*	15.6
23	165.1 *s*			164.8 *s*			164.4 *s*		
Me-16	20.3 *q*	0.87 *d*	6.6	20.3 *q*	0.87 *d*	6.6	20.4 *q*	0.92 *d*	6.6
1′	129.6 *s*			137.7 *s*			137.5 *s*		
2′,6′	130.2 *d*	7.05 *d*	8.4	128.9 *d*	7.18 *d*	7.4	129.1 *d*	7.17 *d*	7.2
3′,5′	115.8 *d*	6.77 *d*	8.4	129.1 *d*	7.31 *dd*	7.4, 7.4	129.0 *d*	7.32 *dd*	7.2, 7.2
4′	155.2 *s*			126.9 *q*	7.23 *dd*	7.4, 7.4	127.1 *d*	7.24 *dd*	7.2, 7.2
NH		5.53 *br s*			5.50 *br s*			5.55 *br s*	

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

[b] Multiplicities were determined by DEPT spectra.

(Capasso et al., 1991) in the absence of the HC(7)–OH and H_2C=C(6) signals, whereas two broad singlets at δ 5.38 and 1.77, typical chemical shift values of an olefinic proton (H-7) and a vinyl methyl group (Me-12) (Pretsch et al., 1989), were present. The same differences were observed in its ^{13}C NMR spectrum, which, in respect to that of **1** (Capasso et al., 1991), showed the absence of secondary hydroxylated and olefinic methylene carbons and the presence of a doublet and a quartet at δ 124.2 and 19.9, which are assigned to C-7 and to the Me-12 group, and the upfield shift ($\Delta\delta$ 11.3) of C-6 δ 140.2 (Breitmaier and Voelter, 1987). In comparison with **4**, the HC(20)–OH signals were absent in the ^1H and ^{13}C NMR spectra of **5**, whereas a complex multiplet and a triplet carbon were observed at δ 2.28 and at δ 34.1, respectively, and assigned to a new methylene group (CH_2-20) by the couplings observed in the COSY and TOCSY and HMQC (Braun et al., 1998) spectra. Another significant difference noted in **5** was the signal pattern of the phenyl residue attached at C-10. In fact, in the ^1H and ^{13}C NMR spectra of **5** we observed the system typical of a *para*-hydroxy substituted phenyl ring with two couples of doublets (J = 8.4 Hz) at δ 7.05 and 6.77, due, respectively, to the two protons (H-2′ and H-6′) *ortho*-positioned to the carbon attached at C-10, and the other two upfield shifted ($\Delta\delta$ 0.54) aromatic protons (H-3′ and H-5′) *ortho*-positioned in respect to an oxygenated group (Pretsch et al., 1989). These protons correlated with the corresponding coincident carbon couples observed at the expected chemical shift values of δ 130.2 (C-2′ and C-6′) and δ 115.8 (C-3′ and C-5′). The latter resonated upfield shifted ($\Delta\delta$ 13.1) with respect to **4** as well as the C-1′ ($\Delta\delta$ 7.8) singlet appearing at δ 129.6, respectively, typical chemical shift values for aromatic carbons *ortho*- and *para*-located to a hydroxy group; the C-4′ singlet appeared significantly downfield shifted ($\Delta\delta$ 28.1) at δ 155.2, which is the characteristic value of a quaternary aromatic carbon bonded to a hydroxy group (Breitmaier and Voelter, 1987).

On this basis and in addition, the correlations observed in the COSY, TOCSY and HMQC spectra, which also allowed us to assign the chemical shifts to all the protons and corresponding carbons (Table 1), the structure of a 20-deoxycytochalasin T bearing a *p*-hydroxybenzyl group at C-3 was assigned to cytochalasin Z1 (**5**).

The structure was confirmed by the ^1H, ^{13}C long-range correlations and NOEs observed in the HMBC and NOESY spectra (Braun et al., 1998) the most significant of which are reported in Table 2.

The structure assigned to **5** was also supported by the data from the mass spectra. In fact, in the EI MS spectrum beside the molecular ion $[M]^+$ at m/z 463, some significant fragmentation peaks, which are generated by successive losses of methyl H_2O and *p*-hydroxybenzyl residues from the parent ion, were observed at m/z 448, 430 and 338, respectively. Alternatively, the molecular ion yielded the ion at m/z 356 by the loss of the *p*-hydroxybenzyl residue, which also generated the peak $[C_7H_6OH]^+$ at m/z 107. The ES MS spectrum showed clustered potassium and sodium and the pseudomolecular $[M+H]^+$ ions at m/z 502, 486 and 464, respectively, while in the FAB MS spectrum the pseudomolecular ion $[M+H]^+$ present at m/z 464 generated, by loss of H_2O, the ion at m/z 446.

By preliminary ^1H and ^{13}C NMR investigation, cytochalasin Z2 showed a structure very close to that of cytochalasin T (**4**), but the molecular weight of 479 deduced from EI, ES and FAB mass spectra also indicated a significant difference due to an additional oxygen atom. This was localized, as shown below, in the hydroxymethyl group bonded to the C-6 of the perhydroisoindolyl-1-one moiety. Comparison of the ^1H NMR spectrum of **6** (Table 1) with that of cytochalasin T (Capasso et al., 1991) showed the absence of the singlet at δ 1.75 attributed in **4** to the vinyl methyl group and the presence of a broad singlet at δ 4.15, which is a typical chemical shift value for a hydroxymethyl group bonded to an olefinic carbon (Pretsch et al., 1989). As expected, the ^{13}C NMR spectrum of **6** lacked the quartet due to the vinyl methyl group, which resonated in **4** at δ 21.6, but showed the triplet of a hydroxylated methylene carbon ($HOCH_2$-12) at the characteristic chemical shift value of δ 63.5 (Breitmaier and Voelter, 1987). The correlation observed in the COSY, TOCSY and HMQC confirmed this partial structure contained in **6** and also allowed us to assign the chemical shifts of protons and carbons (Table 1).

Therefore, it is possible to assign the structure of 12-hydroxycytochalasin T to cytochalasin Z2 (**6**). The structure **6** was confirmed by the ^1H, ^{13}C NMR long-range correlations and NOEs observed in HBMC and NOESY spectra, the most significant of which are reported in Table 2.

Finally, the structure assigned to **6** was also supported by the data obtained from its mass spectra. The EI MS spectrum showed the molecular ion at m/z 479 $[M]^+$, which by successive losses of a Me residue and two H_2O molecules yielded significant fragmentation peaks at m/z 464, 446 and 428, respectively, and, alternatively, by loss of the C_7H_7 residue followed by that of two H_2O molecules, the ions at m/z 388, 370 and 352, respectively. The ES MS showed clustered potassium and sodium ions and at m/z 518 and 502, while the pseudomolecular ion $[M+H]^+$ was observed at m/z 480. The latter ion, which was also present in the FAB MS spectrum, generated the ion at m/z 462 by loss of H_2O.

Preliminary spectroscopic investigation (^1H and ^{13}C NMR) of cytochalasin Z3 (**7**) suggested its structural relation with cytochalasin B (**1**), and the same molecular weight of 479. A comparison of the ^1H NMR spectrum of **7** (Table 1) with that of **1** (Capasso et al., 1991) highlighted the increased multiplicity of H-21. It

Table 2
The most significant ^{1}H, ^{13}C NMR long-range correlations and NOE effects observed, respectively, in the HMBC and NOESY spectra of Z1, Z2 and Z3 (**5**, **6** and **7**, respectively)

5		**6**		**7**	
HMBC data					
δC	δH	δC	δH	δC	δH
155.2 (C-4′)	7.05 (H-2′ and/or H-6′), 6.77 (H-3′ and/or H-5′)	143.1 (C-6)	4.15 (H_2-12), 3.41 (H-8), 2.78 (H-4), 1.31 (Me-11)	146.8 (C-21)	2.74 (H-20A), 2.25 (H-20B)
152.6 (C-21)	2.28 (H-20)	127.1 (C-7)	5.95 (H-13), 4.15 (H_2-12), 3.41 (H-8)	122.7 C-22)	7.03 (H-21), 2.74 (H-20A), 2.25 (H-20B)
140.2 (C-6)	2.70 (H-4), 1.77 (H-15B), 1.22 (Me-11)	63.5 (C-12)	5.64 (H-7)	71.9 (C-19)	2.74 (H-20A), 2.25 (H-20B), 1.88 (H-18A)
129.6 (C-1′)	6.77 (H-3′ and/or H-5′), 2.92 (H-10A), 2.81 (H-10B)	33.8 (C-5)	5.64 (H-7), 4.15 (H_2-12), 3.31 (H-3), 2.78 (H-4)	42.7 (C-20)	7.03 (H-21), 5.75 (H-22), 1.88 (H-18A)
124.2 (C-7)	1.77 (Me-12)			37.7 (C-18)	2.74 (H-20A), 0.79 (H-17B)
121.0 (C-22)	2.28 (H-20)				
115.8 (C-3′,5′)	7.05 (H-2′ and/or H-6′)				
34.3 (C-19)	2.28 (H-20)				
34.1 (C-20)	7.17 (H-21), 5.69 (H-22), 1.73 (H-19A), 1.35 (H-19B)				
NOESY data					
Considered	Effects	Considered	Effects	Considered	Effects
7.17 (H-21)	5.69 (H-22), 2.28 (H-20), 1.73 (H-19A)	5.64 (H-7)	4.15 (H_2-12), 3.41 (H-8)	7.03 (H-21)	5.75 (H-22), 2.74 (H-20A), 2.25 (H-20B)
6.77 (H-3′-5′)	5.53 (NH)	4.15 (H_2-12)	5.64 (H-7), 1.33 (Me-11)	5.75 (H-22)	7.03 (H-21), 2.74 (H-20A), 2.25 (H-20B)
5.69 (H-22)	7.17 (H-21), 2.28 (H-20)			3.71 (H-19)	2.74 (H-20A), 2.25 (H-20B), 1.88 (H-18A)
5..38 (H-7)	3.20 (H-8), 1.77 (Me-12)			2.74 (H-20A)	7.03 (H-21), 5.75 (H-22), 3.71 (H-19), 2.25 (H-20B)
2.28 (H-20)	7.17 (H-21), 5.69 (H-22), 1.73 (H-19A), 1.35 (H-19B)			2.25 (H-20B)	7.03 (H-21), 5.75 (H-22), 3.71 (H-19), 2.74 (H-20A)

appeared as a doublet of double doublets ($J = 15.6$, 8.4 and 8.4 Hz) at δ 7.03 and coupled in the COSY spectrum with the other *trans*-positioned olefinic proton H-22, a doublet ($J = 15.6$ Hz) at δ 5.75, and with the two protons H_2C-20, a multiplet and a doublet of double doublets ($J = 8.6$, 8.4 and 8.4 Hz) observed at δ 2.74 and 2.25, respectively (Pretsch et al., 1989). This partial structure resembled the one observed in **5**, but in **7** the two protons of H_2C-20 proved to be coupled to the proton of a secondary carbon bearing a hydroxy group (HOCH-19) resonating at δ 3.71 as a complex multiplet H-19. This, in turn, coupled to the protons of the adjacent methylene group (H_2C-18) observed as multiplets at δ 1.88 and 1.33 (Pretsch et al., 1989). In the HMQC spectrum (Table 1), this proton (H-19) correlated with a secondary hydroxylated carbon at δ 71.9, while the two protons of its adjacent methylene groups (H_2C-20 and H_2C-18) coupled to the carbons present at δ 42.7 and 37.7, respectively. With respect to **1**, the latter (C-18) appeared significantly downfield shifted ($\Delta\delta$ 16.2) (Breitmaier and Voelter, 1987).

On the basis of these data and the other correlations observed in the COSY and HMQC spectra, which also allowed us to assign the chemical shifts of all protons and the carbons (Table 1), the structure of a 20-deoxy-19-hydroxycytochalasin B (**7**) was assigned to cytochalasin Z3.

This structure was consistent with the correlations and NOEs observed in HBMC and NOESY spectra of **7**, the most significant of which are reported in Table 2.

The structure assigned to **7** was also supported by the fragmentation peaks observed in its EI MS spectrum. In fact, the molecular ion at m/z 479 by successive losses of benzyl residue (C_7H_7) and two H_2O and CO molecules generated the ions at m/z 388, 370, 352 and 302, respectively, and alternatively by consecutive loss of two H_2O molecules and a Me residue yielded the ions at m/z 461, 443 and 428, respectively. The benzyl residue produced the significant base peak $[C_7H_7]^+$ at m/z 91.

Cytochalasin Z1 represents the first example of a 24-oxa[14]cytochalasan bearing a *p*-hydroxybenzyl residue at C-3 of the perhydroisoindolyl-1-one moiety, and therefore, differed from the other [14]cytochalasans showing a phenyl, isopropyl or an indol-3-yl residue at C-10 and having a different functionalised macrocyclic ring (Cole and Cox, 1981; Natori and Yahara, 1991; Vurro et al., 1997). The most closely related cytochalasins are phenolchalasins A and B, two 21,23-dioxa[13]cytochalasans with a lactonic macrocyclic ring, produced by *Phomopsis* sp. (Tomoda et al., 1999), pyrichalasin H, a phytotoxic [11]cytochalasan with a carbocyclic macrocyclic ring produced from *Pyricularia grisea* (Nukina, 1987) and phomopsischalasin, an antimicrobial [13]cytochalasan in which the macrocyclic ring arranges into a tricyclic carbocyclic system fused to the perhydroisoindolone unit, produced by an endophytic *Phomopsis* sp. (Horn et al., 1995). The first two fungal cytochalasins and **5** had a *p*-hydroxybenzyl at C-3, which should be biosynthesised from tyrosine, while the latter two had a *p*-methoxybenzyl, which should be derived from tyrosine methyl ether (Natori and Yahara, 1991). Furthermore, cytochalasins Z1 and Z3, structurally related to cytochalasins T and B, respectively, are the first two 24-oxa[14]cytochalasans with a lactonic macrocyclic ring deoxygenated at C-20 (Cole and Cox, 1981; Natori and Yahara, 1991; Vurro et al., 1997). Cytochalasin Z2, closely related to the well known cytochalasin T, is a new 24-oxa-[14] cytochalasan showing for the first time, among all the [11], [13] and [14]cytochalasans, a hydroxymethyl group on the C-6, (Cole and Cox, 1981; Natori and Yahara, 1991; Vurro et al., 1997).

In seedling assays (Fig. 1) on wheat and on tomato, the most active compounds were cytochalasin B (CB), its 21,22-dihydroderivative (diHCB), prepared by $NaBH_4$ reduction of **1** (Bottalico et al., 1990), cytochalasins F and Z3 (CF and CZ3), and deoxaphomin (DEOXA). They were all able to reduce the root length by about 50%. In the puncture assay, only deoxaphomin, at the used concentration, showed the ability to produce small necrotic lesions, whereas no effects were produced in the immersion assay by any of the tested cytochalasins. This, together with the observed phytotoxicity of liquid culture filtrates, could mean that other metabolites are responsible for phytotoxic effects caused by the pathogen. The existing structural correlation of cytochalasins Z1 and Z2 with cytochalasin T, and of cytochalasin Z3 with CB was also observed biologically. The first two were inactive, whereas the other two proved to be active in the root elongation assay. These results are in accordance with those previously described in structure-activity relationship studies, which showed the important role of the hydroxy group at C-7 in conferring biological activity (Bottalico et al., 1990; Capasso et al., 1991; Vurro et al., 1997).

Cytochalasins have been considered as potential mycotoxins. If high level of toxins were really produced in vivo, this could, in practice, make it hazardous to use *P. semeniperda* as a biological control agent against grass weeds. Hence, studies are in progress both to quantify the presence of such toxins in naturally infected seeds, as well as to estimate their stability and impact in the environment. In South Africa, *P. semeniperda* was one of several fungi isolated from leaf spots of grazing oats (*Avena sativa* L.) in association with field outbreaks of diarrhoea, photosensitivity and death in goats, diarrhoea and loss of milk production in dairy cattle. The fungus was highly toxic when fed freely as maize seed culture to ducklings, and as a pure meal drench to goats and sheep (Schneider et al., 1985; Collett et al., 1988), even if there was no evidence that the fungus had mycotoxic properties other than at high dosage of almost pure cultures. Further observations

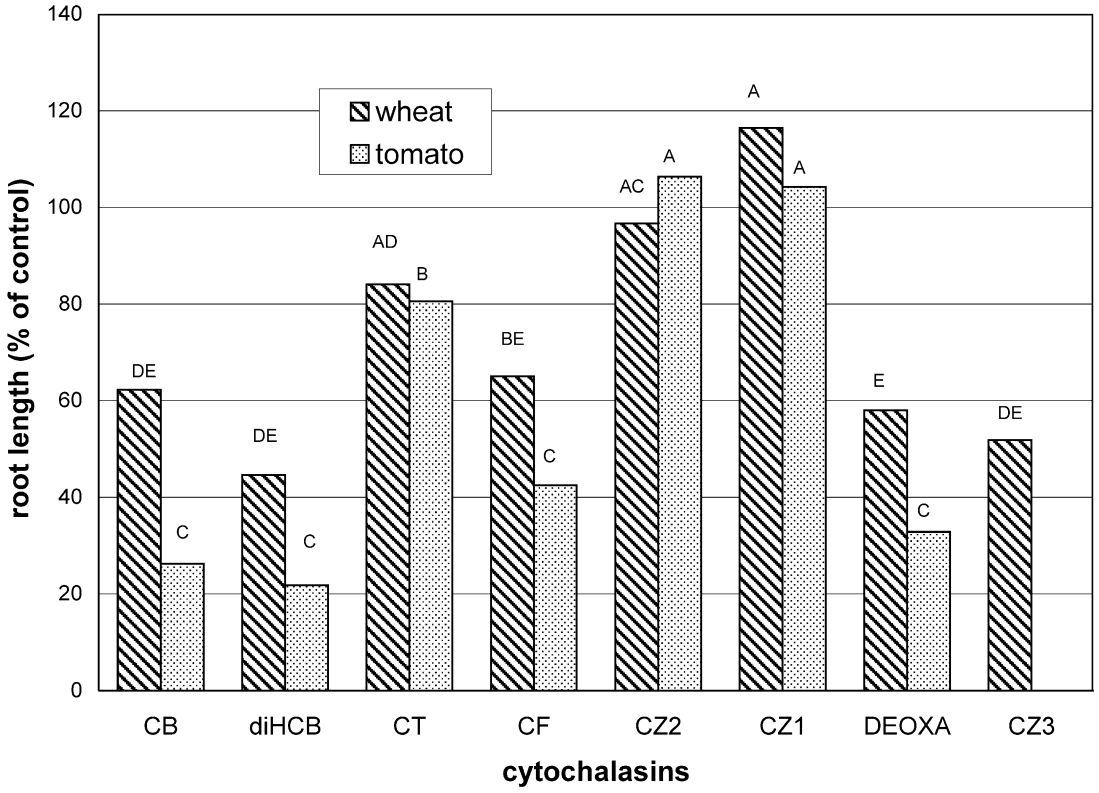


Fig. 1. Effect of cytochalasins on root elongation of tomato and wheat seedlings. Root length was measured 3 days after treatment, and expressed as percentage with respect to the control. Means of root length was 20.5 (±10.6) and 52.1 (±10.9) mm for wheat and tomato, respectively. Values denoted by different letters are significantly different at $P = 0.05$. Abbreviations: CB, CF, CT, CZ1, CZ2 and CZ3 = cytochalasins B, F, T, Z1, Z2 and Z3, respectively; diHCB = 21,22-dihydrocytochalasin B; DEOXA = deoxyphomin.

are also under way to identify the lipophilic phytotoxic compounds that the fungus is able to produce in liquid culture. If the found toxins were proved to be important in the disease caused by the fungus, an interesting alternative strategy could be the use of such metabolites as natural herbicide instead of the living agent.

3. Experimental

3.1. General

IR and UV spectra were determined as neat and in CH_3CN solution, respectively, on a Perkin-Elmer IR FT-1720X spectrometer and a Perkin-Elmer Lambda 3B spectrophotometer. ^1H and ^{13}C NMR spectra were recorded at 500 and 100 MHz, respectively, in $CDCl_3$, on Bruker spectrometers. The solvent was used as internal standard. Carbon multiplicities were determined by DEPT spectra (Braun et al., 1998). DEPT, COSY-45, TOCSY, HMQC, HMBC and NOESY experiments were performed using Bruker microprograms. EI and FABMS were taken at 70 eV and glycerol/thioglycerol using Cs as bombarding atoms on a Fisons Trio-2000 and a Fisons ProSpec spectrometer, respectively. Electrospray 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) plates; the spots were visualised by exposure to UV radiation and/or by spraying with 10% H_2SO_4 in methanol and then with 5% phosphomolybdic acid in methanol, followed by heating at 110 °C for 10 min. Column chromatography: silica gel (Merck, Kieselgel 60, 0.063–0.20 mm). Solvent systems: (A) $CHCl_3$–*iso*-PrOH (9:1), (B) EtOAc–*n*-hexane (7:3); (C) $CHCl_3$–*iso*-PrOH (93:7).

3.2. Production of cytochalasins

A strain of *P. semeniperda* was kindly supplied by Professor Richard W. Medd, Department of Agriculture, Orange Agricultural Institute, Orange, NSW, Australia, and kept on PDA (potato-dextrose-agar) Petri dishes. For the production of toxins the fungus was grown on steamed and autoclaved wheat kernels at 25 °C for 4 weeks.

3.3. Bioassay methods

3.3.1. Wheat and tomato seedlings assay

Wheat (*Triticum durum* L.) and tomato (*Lycopersicon esculentum* L.) seeds were surface sterilised with NaClO

(2,5%) (20 and 10 min, respectively), thoroughly washed with sterile water, and left to germinate on wet filter paper in glass Petri dishes in the dark, at 25 °C. 10 seeds were then transferred into Petri dishes on paper filters wetted with 2 ml of toxin solutions (1% of MeOH). Dishes were placed in a growth chamber at 25 °C for 3 days under fluorescent lights (12 h light/12 h dark) and then the rootlet lengths were measured. All compounds were assayed at 10^{-4} M with 3 replicates. Results are expressed in Fig. 1. The data were subjected to analysis of variance and the means were compared by Duncan's multiple range test.

3.3.2. Seedling immersion assay

All compounds were assayed on wheat seedlings. Metabolites were dissolved in a small volume of MeOH (1% of the final solution) and then brought up to the final concentration (10^{-4} M) with distilled water. Plants were grown in vermiculite pots in a greenhouse. Seedlings were washed and roots were immersed in 2 ml of test solution and kept in a growth chamber at 25 °C for 24 h under continuous fluorescent light. Plants (three replicates for each treatment) were then transferred into distilled water and kept for a further 48 h.

3.3.3. Leaf puncture assay

Wheat leaves were detached from plants and placed in a moist chamber under fluorescent lights. Droplets (15 μl) of solutions containing the toxins (4×10^{-4}) were applied on the leaf surface, after being punctured with a needle. Leaves were observed daily for symptom appearance, up to 6 days.

3.4. Extraction and purification of cytochalasins

Wheat cultures of *P. semeniperda* were dried and finely minced; 100 g of dried material was extracted with a MeOH–H_2O (1% NaCl) mixture (55:45 v/v), defatted by *n*-hexane and then extracted with CH_2Cl_2. The organic extracts were combined, dried (Na_2SO_4) and evaporated under reduced pressure yielding a solid mixed with a brown oil (476 mg) showing a significant phytotoxic activity. The mixture was washed with little aliquots (5×1 ml) of CH_3OH. The solid residue was soluble in chloroform–methanol (1:1) and essentially contained cytochalasin B as shown by TLC analysis carried out in comparison with an authentic sample of the toxin (R_f 0.71 and 0.47, using the solvent systems A and B, respectively). The solid was crystallised twice from EtOAc–*n*-hexane (1:5 *v*/*v*) giving white needles (221 mg) of cytochalasin B (**1**). The mother liquors (23.2 and 6.7 mg, respectively) of cytochalasin B crystallisation were combined with the initial methanol fraction (total 251.3 mg) and fractionated by column chromatography (eluent A), yielding nine groups of homogeneous fractions. The residues of the fourth (100.1 mg) and fifth (98.1 mg) fraction groups were crystallised as described above to give other aliquots (67.3 and 75.8 mg, respectively, amounting to a total of 364.1 mg) of cytochalasin B. The mother liquors of crystallisation (16.2 mg) of the fourth fraction and the residue (4.1 mg) of the third fraction of the initial column were further purified by preparative TLC (eluent B) to afford two pure metabolites as amorphous solids (R_f 0.68 and 0.81, respectively). The less polar of the latter (1.3 mg) proved to be the cytochalasin T (**4**). The other metabolite (1.1 mg), proved to be new and was named cytochalasin Z1 (**5**). The mother liquors of crystallisation (16.2 mg) of the fifth fraction were similarly purified to give two other metabolites (R_f 0.32 and 0.54) as pure amorphous solids (1.2 and 2.0 mg, respectively), which proved to be deoxaphomin (**2**) and the cytochalasin F (**3**). Finally, purifying the residue (15.2 mg) of the sixth fraction of the initial column by preparative TLC (eluent C) gave a pure amorphous solid (R_f 0.50, 1.1 mg) which proved to be another new cytochalasan named cytochalasin Z2 (**6**).

When the same purification procedure was applied to the organic extract (3.17 g) obtained from 780 g of the same solid culture of *P. semeniperda*, further amounts of crystalline cytochalasin B (2.3 g) as well as of the other cytochalasins F, T Z1, Z2 and deoxyphomin (3.4, 4.3, 2.5, 10 and 12 mg, respectively) were obtained.

Furthermore, purification of the sixth fraction residue (23 mg) of the initial column, which contained metabolites with intermediate polarity between cytochalasin Z1 and deoxyphomin (R_f 0.81 and 0.32, respectively), by two preparative TLC steps (eluents C and B respectively) allowed us to isolate another amorphous homogeneous solid (1.3 mg), which proved to be another new cytochalasan named cytochalasin Z3 (**7**).

3.5. Cytochalasin Z1 (*5*)

Compound **5**: IR ν_{max} cm^{-1} 3460, 1716, 1651, 1516, 1462, 1295, 1257; UV λ_{max} nm (log ε): 275 (2.61); ^1H and ^{13}C NMR: Table 1; EI MS (rel. int.) *m*/*z*: 463 $[M]^+$ (6), 448 $[M–Me]^+$ (8), 430 $[M–Me–H_2O]^+$ (6), 356 $[M–C_7H_6OH]^+$ (6), 338 $[M–H_2O–Me–C_7H_6OH]^+$ (9), 107 $[C_7H_6OH]^+$ (24), 55 (100); ES MS (rel. int.) *m*/*z*: 502 $[M+K]^+$, 486 $[M+Na]^+$, 464 $[M+H]^+$; FAB MS (rel. int.) *m*/*z*: 464 $[M+H]^+$ (100), 446 $[M+H–H_2O]^+$ (9).

3.6. Cytochalasin Z1 (*6*)

Compound **6**: IR ν_{max} cm^{-1} 3415, 1720, 1651, 1510, 1445, 1260, 1218; UV λ_{max} nm: <220; ^1H and ^{13}C NMR: Table 1; EI MS (rel. int.) *m*/*z*: 479 $[M]^+$ (1), 464 $[M–Me]^+$ (4), 446 $[M–Me–H_2O]^+$ (1), 428 $[M–Me–2\times H_2O]^+$ (2), 388 $[M–C_7H_7]^+$ (7), 370 $[M–C_7H_7–H_2O]^+$ (8), 352 $[M–C_7H_7–2xH_2O]^+$ (5), 91 $[C_7H_7]^+$ (100); ES MS (rel. int.) *m*/*z*: 518 $[M+K]^+$, 502

$[M+Na]^+$, 480 $[M+H]^+$; FAB MS (rel. int.) m/z: 480 $[M+H]^+$ (100), 462 $[M+H-H_2O]^+$ (32).

3.7. Cytochalasin Z3 (7)

Compound **7**: IR ν_{max} cm^{-1} 3377, 1708, 1644, 1496, 1455, 1263; UV λ_{max} nm: <220; ^1H and ^{13}C NMR: Table 1; EI MS (rel. int.) m/z: 480 $[M+H]^+$ (0.2), 479 $[M]^+$ (0.1), 461 $[M-H_2O]^+$ (0.3), 443 $[M-2\times H_2O]^+$ (0.5), 428 $[M-2xH_2O-Me]^+$ (0.4), 389 $[M+H-C_7H_7]^+$ (1), 388 $[M-C_7H_7]^+$ (5), 370 $[M-C_7H_7-H_2O]^+$ (4), 352 $[M-C_7H_7-2\times H_2O]^+$ (3), 302 $[M-C_7H_7-2xH_2O-CO]^+$ (8), 91 $[C_7H_7]^+$ (100).

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