Cytochalasins Z4, Z5, and Z6, Three New 24-Oxa[14]cytochalasans Produced by *Phoma exigua* var. *heteromorpha*†

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Cytochalasins Z4, Z5, and Z6, three new cytochalasans, were isolated from the wheat culture of *Phoma exigua* var. *heteromorpha* together with the known cytochalasins A, B, F, T, Z2, and Z3, 7-*O*-acetylcytochalasin B, and deoxaphomin. Z2 and Z3, together with Z1, have recently been described as new 24-oxa[14]cytochalasans produced by *Pyrenophora semeniperda*, a fungus proposed for biological control of grass weeds. All three new cytochalasins were characterized as 24-oxa[14]cytochalasans by extensive use of NMR and MS techniques. Cytochalasins Z4 and Z5 proved to be structurally related to cytochalasin B, whereas Z6 was related to cytochalasin F. When assayed on tomato seedlings at 10^{-4} M, Z6 caused a very low inhibition of rootlet elongation (30%), whereas Z4 and Z5 were almost inactive. On brine shrimp, at the same concentration, only Z5 had a minor effect, with 20% mortality, whereas the other two metabolites proved to be inactive.

We recently isolated three new cytochalasins, named Z1, Z2, and Z3, from wheat cultures of *Pyrenophora semeniperda* (Brittlebank & Adam) Shoemaker, together with the already known cytochalasins B, T, and F and deoxyphomin.[1] *P. semeniperda* is a seed-borne pathogen proposed as a bioherbicide[2] for biological control of grass weeds. Cytochalasins constitute a large group of metabolites produced by several different fungal species, and, although the first metabolite was discovered more than 30 years ago, they are still widely studied for their interesting biological properties and potential applications as pharmaceutical, antibiotic, or herbicidal products. Considering the potential applications and the availability of large amounts of solid cultures of *Phoma exigua* var. *heteromopha* (Schulzer *et* Sacc.) Noordeloos *et* Boerema, a good producer of cytochalasins,[3] we decided to look for new cytochalasins yielded by this fungus. This work describes the chemical characterization of cytochalasins Z4, Z5, and Z6, three new cytochalasins isolated together with cytochalasins A, B, F, T, Z2, and Z3, 7-*O*-acetylcytochalasin B, and deoxaphomin[1,3] from the organic extract of a large amount of *P. exigua* var. *heteromorpha* wheat kernel culture.

Results and Discussion

Fungal cultures of *P. exigua* var. *heteromorpha*, grown on wheat kernels, were first extracted with a water−methanol mixture, defatted, and re-extracted with methylene chloride, and then dried, yielding an abundant solid residue together with a brown oil (2.80 g/kg). The solid was washed with small aliquots of methanol, and the residue obtained, crystallized from EtOAc−*n*-hexane, gave the main metabolite, cytochalasin B (**1**, 2.12 g/kg).[4] The mother liquors of the cytochalasin B crystallization were combined with the methanolic washes and fractionated by silica gel column chromatography (eluent, $CHCl_3$−*i*-PrOH, 9:1), as previously described.[5] Eight groups of homogeneous fractions were collected and compared by TLC (eluents, $CHCl_3$−*i*-PrOH, 9:1, and EtOAc−*n*-hexane, 7:3) with several standard cytochalasins. Among others, cytochalasin B proved to be the predominant metabolite in the extract, and some of them (A, F, T, Z2, Z3, 7-*O*-acetylcytoochalasin B, and deoxaphomin) have already been reported. Fractions seven and eight were further purified by multiple steps of preparative TLC (see Experimental Section) on silica gel and reversed-phase plates. They yielded additional amounts of cytochalasins Z2 (**4**) and Z3 (**5**) (11.0 and 10.2 mg; 4.4 and 4.1 mg/kg dry weight, respectively, in each fraction), recently isolated from *P. semeniperda* culture extracts,[1] together with three undescribed 24-oxa[14]cytochalasans. Obtained as amorphous homogeneous solids (3.5, 0.7, and 0.2 mg; 1.43, 0.28, and 0.08 mg/kg dry weight, respectively, in each fraction), we named the last three compounds cytochalasins Z4, Z5, and Z6 (**6**, **7**, and **8**, respectively).

Preliminary ^{1}H and ^{13}C NMR investigations showed that Z4 and Z5 (**6** and **7**) are structurally related to cytochalasin B (**1**), whereas Z6 (**8**) is related to cytochalasin F (**2**). Both Z4 and Z5 have a molecular weight of 495.2622, as established by HREI and ES mass spectra, corresponding to a molecular formula of $C_{29}H_{37}NO_6$. They both differed from cytochalasin B by the presence of an additional oxygen atom of a phenolic hydroxy group located on the benzyl group, which, in turn, is attached to C-3 of the perhydroisoindolyl-1-one residue. COSY and HSQC spectra[6] showed that the proton and carbon signals stemming from the macrocyclic ring and the perhydroisoindolyl residue were very similar to those described for cytochalasin B (**1**).[5] Therefore, the assignment of the chemical shifts of all the protons and carbons could be achieved (Table 1). The main difference was the signal pattern of the monosubstituted hydroxyphenyl ring attached to C-10. In fact, the ^{1}H NMR spectrum of **6** exhibited the presence of two doublets (J = 8.0 Hz) at δ 7.05 and 6.81 (H-6′ and H-3′, respectively) and two doublets of doublets (J = 8.0 and 8.0 Hz) at δ 7.09 and 6.83 (H-4′ and H-5′, respectively), typical of an *o*-hydroxy-substituted phenyl residue.[7] The spectrum of **7** showed the presence of two doublets (J = 8.6 Hz) at δ 7.02 and 6.76 (H-2′, H-6′ and H3′, H-5′, respectively), typical of a *p*-hydroxy-substituted phenyl residue,[7] in agreement with those observed in the spectrum of cytochalasin Z1 (**3**).[1] As

† Dedicated to the memory of Prof. Franco Tatò.

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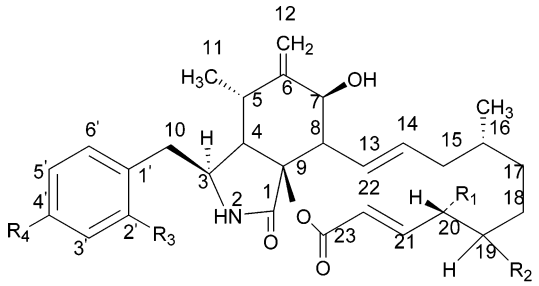
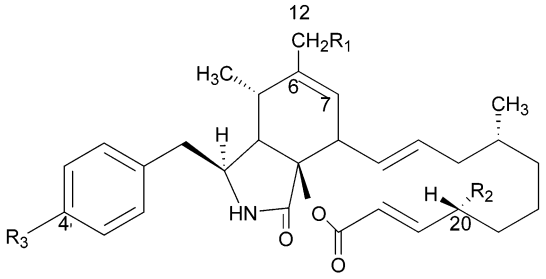


Cytochalasin B, **1** R_1=OH, R_2=R_3=R_4=H

Cytochalasin Z3, **5** R_1=R_3=R_4=H, R_2=OH

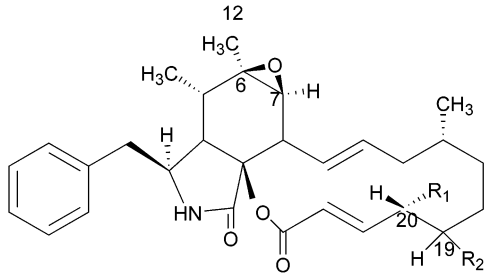
Cytochalasin Z4, **6** R_1=R_3=OH, R_2=R_4=H,

Cytochalasin Z5, **7** R_1=R_4=OH, R_2=R_3=H



Cytochalasin Z1, **3** R_1=R_2=H, R_3=OH

Cytochalasin Z2, **4** R_1=R_2=OH, R_3=H



Cytochalasin F, **2** R_1=OH, R_2=H

Cytochalasin Z6, **8** R_1=H, R_2=OH

expected, the ^{13}C NMR spectrum of **6** showed hydroxylated and alkylated quaternary (C-2′ and C-1′) aromatic carbons at the typical chemical shift values of δ 154.4 and 123.8, respectively, and those of the *ortho*-, *meta*-, and *para*- (C-3′, C-4′, and C-6′ and C-5′, respectively) positioned carbons with respect to C-2′ at δ 115.8, 128.4, and 131.5 and 120.7, respectively.[8] By analogy, the ^{13}C NMR spectrum of **7** showed the hydroxylated and alkylated quaternary carbons (C-4′ and C-1′) and the pairs of protonated carbons (C-2′,6′ and C-3′,5′) at the typical chemical shift values of δ 154.5, 129.1, 130.6, and 115.8, respectively.[8] These data were in perfect agreement with those observed in **3**.[1]

On the basis of these results the cytochalasin B structure, but with a *ortho*- or *para*-hydroxybenzyl group at C-3, was assigned to Z4 and Z5, respectively. Both structures were confirmed by the HMBC ^{1}H−^{13}C long-range correlations observed and by NOESY spectra,[6] the most significant of which are reported in Table 2.

The structure assigned to cytochalasin Z4 (**6**) was further supported by the chemical shift of the broad singlet of the NH proton at δ 6.20. As compared to cytochalasin B (**1**) and other 24-oxa[14]cytochalasans[1,3,5] under the same conditions, it shows a significant downfield shift ($\Delta\delta$ 0.60). This unusual shift is due to hydrogen bonding between the NH of the perhydroisoindolyl-1-one moiety and the hydroxy group located at C-2. Inspection of a Dreiding model of **6** also supported this conclusion.

The structure assigned to cytochalasins **6** and **7** was confirmed by the EI and ES mass data. Both showed the same molecular ion at *m*/*z* 495 and the same fragmentation ion pattern. In fact, by alternative or successive losses of two H_2O molecules and the hydroxybenzyl residue, the parent ion generated ions at *m*/*z* 477, 459, 388, 370, and 352.[7] The ES mass spectra of both cytochalasins (positive mode) showed potassium and sodium adducts and the pseudomolecular ion $[M + H]^+$ at *m*/*z* 534, 518, and 496, respectively. In **6**, the ion at *m*/*z* 389 was also generated by loss of the *p*-hydroxybenzyl residue; the ES mass spectrum recorded (negative mode) showed the pseudomolecular ion $[M - H]^-$ at *m*/*z* 494 and ions at *m*/*z* 476 and 458 produced by losses of H_2O molecules.

By preliminary spectroscopic investigation (MS and ^{1}H and ^{13}C NMR), Z6 (**8**) showed the same molecular ion at *m*/*z* 479 and a structure very similar to that of cytochalasin F (**2**), previously isolated from the same fungus[5] and, more recently, from *P. semeniperda* culture extracts.[1] In particular, the ^{1}H NMR spectrum (Table 1) of **8**, compared to that of **2**,[5] showed the expected doublet of doublet (J = 10.1 and 5.4 Hz), doublet (J = 5.4 Hz), and singlet at δ 3.06 (H-8), 2.73 (H-7), and 1.22 (Me-12), respectively, all typical for a suitably trisubstituted oxiran ring.[7] In the HSQC spectrum these protons correlated with corresponding carbons resonating at typical chemical shift values and agreed with values recorded for **2** at δ 61.0 (C-7), 46.4 (C-8), and 19.7 (C-12).[5] As expected, the oxygenated quaternary carbons (C-6) appeared at δ 57.3.[5,8] The only difference between the ^{1}H NMR spectra of both cytochalasins (**8** and **2**) was the increased mutiplicity of H-21, which resonated at δ 6.97 (ddd; J = 15.8, 10.5, and 5.5 Hz) and showed COSY correlations to H-22 and H_2-20, as observed for cytochalasin Z3 (**5**).[1] H_2-20 protons, as observed in **5**,[1] coupled with the proton of the adjacent oxymethine, a complex multiplet at δ 3.74, which in turn was coupled to the protons of the adjacent C-18 methylene group at δ 1.85 and 1.28 (J = 13.7, 13.6, and 5.1 Hz). This partial structure was supported by correlations observed in the HSQC spectrum, which allowed the assignment of the chemical shifts to the corresponding carbons (Table 1) in agreement with the values recorded for **5**.[1,8] Chemical shift differences between compounds **8** and **2** were similar to those observed for **5** and **1**. The structure of 20-deoxy-19-hydroxycytochalasin F (**8**) was assigned to cytochalasin Z6 on the basis of these data and others from COSY and HSQC spectra. They also allowed assignment of the chemical shifts of all protons and corresponding carbons (Table 1).

The structure was consistent with correlations and NOEs observed in HBMC and NOESY spectra of **8**, the most significant of which are reported in Table 2. The structure assigned to **8** was also supported by MS spectra in a manner similar to that described above (see Experimental Section).

Cytochalasins Z4 and Z5 are structurally related to cytochalasin B, the main toxin produced by both *P. exigua* var. *heteromorpha* and *P. semeniperda*, as well as other important toxigenic fungi.[3,9,10] Together with the recently described Z1 (**3**), Z5 (**7**) represents a further example of a 24-oxa[14]cytochalasan bearing a *p*-hydroxybenzyl residue at the C-3 of the perhydroisoindolyl-1-one moiety, whereas,

Table 1. ^{1}H and ^{13}C NMR Spectral Data of Cytochalasins Z4, Z5, and Z6 (**6**, **7**, and **8**, respectively)[a,b]

	6			**7**			**8**		
C	δ_C[c]	δ_H	*J* (Hz)	δ_C[c]	δ_H	*J* (Hz)	δ_C[c]	δ_H	*J* (Hz)
1	171.7 C			171.1 C			171.1 C		
3	53.3 CH	3.38 ddd	(8.6, 5.6, 4.2)	53.4 CH	3.29 m		54.3 CH	3.67 dd	(7.3, 5.9)
4	49.5 CH	2.84 dd	(5.6, 4.4)	49.2 CH	2.81 dd	(4.8, 3.2)	49.3 CH	2.81 m	
5	33.0 CH	3.31 m		31.8 CH	3.31 m		35.8 CH	2.32 m	
6	148.4 C			148.2 C			57.3 C		
7	68.9 CH	3.89 d	(10.8)	69.3 CH	3.88 d	(11.2)	61.0 CH	2.73 d	(5.4)
8	49.4 CH	3.29 dd	(10.8, 10.4)	49.5 CH	3.31 dd	(11.2, 10.1)	46.4 CH	3.06 dd	(10.1, 5.4)
9	83.1 C			82.7 C			84.4 C		
10	38.6 CH_2	2.94 dd	(13.6, 8.6)	43.2 CH_2	2.78 dd	(13.6, 4.4)	45.0 CH_2	2.84 d	(13.3)
		2.82 dd	(13.6, 4.2)		2.71 dd	(13.6, 8.7)		2.78 dd	(13.3, 5.9)
11	14.2 CH_3	1.12 d (3H)	(6.6)	14.1 CH_3	1.11 d (3H)	(6.8)	13.0 CH_3	1.04 d (3H)	(7.3)
12	114.6 CH_2	5.39 br s		114.7 CH_2	5.39 br s		19.7 CH_3	1.22 s	
		5.16 br s			5.17 br s				
13	126.3 CH	5.92 ddd	(15.1, 10.4, 1.8)	126.3 CH	5.93 ddd	(15.2, 10.1, 1.6)	126.5 CH	6.11 ddd	(14.8, 10.1, 1.9)
14	138.1 CH	5.45 ddd	(15.1, 10.5, 3.9)	138.3 CH	5.45 ddd	(15.2, 10.9, 3.5)	135.7 CH	5.30 ddd	(14.8, 10.9, 3.5)
15	41.7 CH_2	2.14 br d	(13.2, 1.8)	41.8 CH_2	2.12 br d	(12.6, 1.6)	41.5 CH_2	2.15 br dd	(13.6, 1.9)
		1.72 ddd	(13.2, 11.1, 10.5)		1.74 ddd	(12.6, 11.2,10.9)		1.71 m	
16	31.9 CH	1.30 m		32.8 CH	1.30 m		33.3 CH	1.32 m	
17	35.0 CH_2	1.65 m		35.0 CH_2	1.67 m		31.1 CH_2	1.62 m	
		0.65 m			0.65 m			0.73 m	
18	20.2 CH_2	1.44 m		20.2 CH_2	1.47 m		37.8 CH_2	1.85 ddd	(13.7, 13.6, 5.1)
		1.30 m			1.26 m			1.28 m	
19	34.8 CH_2	1.88 m		34.9 CH_2	1.93 m		71.5 CH	3.74 m	
		1.60 m			1.61 m				
20	70.9 CH	4.46 m		70.9 CH	4.52 m		42.9 CH_2	2.81 m	
								2.21 ddd	(12.4, 10.5, 10.5)
21	152.2 CH	7.01 dd	(15.7, 5.6)	152.7 CH	7.01 dd	(15.7, 6.3)	146.7 CH	6.97 ddd	(15.8, 10.5, 5.5)
22	119.3 CH	5.86 d	(15.7)	119.2 CH	5.87 dd	(15.7, 1.5)	122.3 CH	5.72 d	(15.8)
23	164.6 C			164.4 C			164.4 C		
Me-C-16	20.3 CH_3	0.88 d	(6.5)	20.4 CH_3	0.89 d	(6.4)	20.0 CH_3	0.88 d	(6.6)
1′	123.8 C			129.1 C			136.9 C		
2′	154.4 C			130.6 CH	7.02 d	(8.6)	129.1 CH	7.15 d	(7.0)
3′	115.8 CH	6.81 d	(8.0)	115.8 CH	6.76 d	(8.6)	128.9 CH	7.33 dd	(7.0, 7.0)
4′	128.4 CH	7.09 dd	(8.0, 8.0)	154.5 C			127.0 CH	7.25 dd	(7.0, 7.0)
5′	120.7 CH	6.83 dd	(8.0, 8.0)	115.8 CH	6.76 d	(8.6)	128.9 CH	7.33 dd	(7.0, 7.0)
6′	131.5 CH	7.05 d	(8.0)	130.6 CH	7.02 d	(8.6)	129.1 CH	7.15 d	(7.0)
NH		6.20 br s			5.61 br s			5.73 br s	

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

in the same group, Z4 (**6**) is the first example of a compound bearing an *o*-hydroxybenzyl residue attached to C-3. Therefore, both cytochalasins differ from the other [14]-cytochalasans because of a phenyl, isopropyl, or indol-3-yl residue at the C-10 and a differently functionalized macrocyclic ring.[3,9,10] The most closely related cytochalasins are phenolchalasins A and B, two 21,23-dioxa[13]cytochalasans having a lactonic macrocyclic ring produced by *Phomopsis* sp;[11] pyrichalasin H, a phytotoxic [11]cytochalasan with a carbocyclic macrocyclic ring produced by *Pyricularia grisea*;[12] and phomopsischalasin, an antimicrobial [13]cytochalasan with a macrocyclic ring arranged into a tricyclic carbocyclic system fused to the perhydroisoindolone unit, produced by an endophytic *Phomopsis* sp.[13] The phenolchalasin A and phomopsischalasin, together with **3** and **7**, have a *p*-hydroxybenzyl at C-3, which should be biosynthesized from tyrosine, whereas phenolchalasin B and pyrichalasin H have a *p*-methoxybenzyl, which should derive from a tyrosine methyl ether.[10] With regard to the *o*-hydroxybenzyl residue attached to the C-3 of Z4 (**6**), a different and new biosynthetic origin should be hypothesized. Furthermore, Z6 (**8**) is the first 24-oxa[14]cytochalasan showing the epoxy group located between C-6 and C-7 of the perhydroisondolyl-1-one residue, the deoxygenation of C-20, and the hydroxylation of C-19, as already observed for Z3.[3,9,10]

In a tomato seedling assay, at 10^{-4} M, only Z6 proved to be slightly active, causing 30% inhibition of root elongation, whereas Z4 and Z5 were inactive. When assayed at the same concentration on brine shrimp, only Z5 caused a quite low mortality of larvae (21%), whereas Z4 and Z6 were both inactive.

The results of structure−activity relationship studies[3,5,14] and recent test results regarding the phytotoxicity of cytochalasin B, its 21,22-dihydro derivative, cytochalasins F, Z1, Z2, and Z3, and deoxaphomin[1] suggest the important role of the hydroxy group at C-7 in conferring the biological activity. This structural feature is present in Z4 and Z5 but not in Z6. Therefore, the lack of phytotoxicity in **6** and **7** is probably due to the *ortho*- or *para*-hydroxy substitution of the benzyl residue attached at C-3. Despite the limited activity of Z6, the presence of an unsubstituted phenyl residue attached at C-10, the most common substituent in this group of natural products, is important for biological activity. This is in accordance with the activity shown by cytochalasin F in the same test[1] and with the results described previously.[3,5,14]

Table 2. Significant HMBC Correlations and NOE Effects Observed in the NMR Spectra of Z4, Z5, and Z6 (**6**, **7**, and **8**, respectively)

HMBC Data

6		**7**		**8**	
δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
154.4 (C-2′)	7.09 (H-4′), 7.05 (H-6′), 2.94 (H-10), 2.82 (H-10′)	154.5 (C-4′)	7.02 (H-2′ and/or H-6′), 6.76 (H-3′ and/or H-5′)	61.0 (C-7)	1.22 (Me-12)
131.5 (C-6′)	7.09 (H-4′), 6.83 (H-5′)	129.1 (C-1′)	6.76 (H-3′ and/or H-5′), 2.78 (H-10), 2.71 (H-10′)	57.3 (C-6)	2.32 (H-5), 1.22 (Me-12), 1.04 (Me-11)
128.4 (C-4′)	7.05 (H-6′), 6.83 (H-5′)	115.8 (C-3′, C-5′)	7.02 (H-2′ and/or H-6′)	35.8 (C-5)	1.22 (Me-12), 1.04 (Me-11)
123.8 (C-1′)	2.94 (H-10), 2.82 (H-10′)	43.2 (C-10)	7.02 (H-2′ and/or H-6′)	164.4 (C-23)	5.72 (H-22)
120.7 (C-5′)	7.05 (H-6′), 6.81 (H-3′)				
115.8 (C-3′)	6.83 (H-5′)				

NOESY Data

6		**7**		**8**	
δ_H signal	correlation to δ_H	δ_H signal	correlation to δ_H	δ_H signal	correlation to δ_H
7.09 (H-4′)	6.83 (H-5′), 6.81 (H-3′)	7.02 (H-2′, H-6′)	6.76 (H-3′ and/or H-5′), 3.29 (H-3), 2.78 (H-10), 2.71 (H-10′)	6.97 (H-21)	5.72 (H-22), 3.74 (H-19), 2.81 (H-20)
6.83 (H-5′)	7.09 (H-4′)	6.76 (H-3′, H-5′)	7.02 (H-2′ and/or H-6′)	5.72 (H-22)	6.97 (H-21), 3.74 (H-19), 2.81 (H-20), 2.21 H-20′)
6.81 (H-3′)	7.09 (H-4′)	3.29 (H-3)	7.02 (H-2′ and/or H-6′)	3.74 (H-19)	6.97 (H-21), 5.72 (H-22), 2.81 (H-20)
		2.78 (H-10)	7.02 (H-2′ and/or H-6′)	3.67 (H-3)	1.22 (Me-12), 1.04 (Me-11)
		2.71 (H-10′)	7.02 (H-2′ and/or H-6′)	2.73 (H-7)	1.22 (Me-12)
				2.81 (H-20)	6.97 (H-21), 5.72 (H-22), 3.74 (H-19), 2.21 H-20′)
				2.21 H-20′)	5.72 (H-22)
				1.22 (Me-12)	3.67 (H-3), 2.73 (H-7)
				1.04 (Me-11)	3.67 (H-3)

Experimental Section

General Experimental Procedures. Optical rotation was measured in $CHCl_3$ solution on a JASCO DIP-370 digital polarimeter; IR (neat) and UV (MeOH) spectra were determined on a Bio-Rad Win FT-IR spectrometer and a Shimadzu UV-1601 UV−visible spectrophotometer, respectively. ^1H and ^{13}C NMR spectra were recorded at 500, 400, or 300 MHz and at 125, 100, or 75 MHz, respectively, in $CDCl_3$, on Bruker spectrometers. The same solvent was used as internal standard. Carbon multiplicities were determined by DEPT spectra.[6] DEPT, COSY-45, HSQC, HMBC, and NOESY experiments[6] were performed using Bruker microprograms. EI and HREIMS were taken 70 eV and on a Fisons Trio-2000 and a Fisons ProSpec spectrometer, respectively. Electrospray MS were recorded on a Perkin-Elmer API 100 LC-MS; a probe 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, F254, 0.25 and 0.5 mm, respectively) or on reversed-phase (Whatmam, Stratocrom KC-18, 0.20 mm) plates. The spots were visualized by exposure to UV radiation and/or by spraying with 10% H_2SO_4 in MeOH and then with 5% phosphomolybdic acid in MeOH, followed by heating at 110 °C for 10 min. Column chromatography was performed on silica gel (Merck, Kieselgel 60, 0.063−0.20 mm).

Fungal Strain. The strain of *P. exigua* var. *heteromorpha* used in this study was isolated in 1985 from necrotic spots of oleander (*Nerium oleander* L.) leaves grown in a nursery near Bari (Italy) and deposited in the fungal collection both of ISPA (ITEM 330) and of Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands (CBS 548.90).

Production, Extraction, and Purification of Cytochalasins Z4, Z5, and Z6 (6, 7, and 8). The fungus was grown on autoclaved wheat kernels at 25 °C for 28 days. Wheat cultures were dried and finely minced; 2.5 kg 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 as previously reported.[4] The organic extracts were combined, dried (Na_2SO_4), and evaporated under reduced pressure, yielding a solid mixed with a brown oil (7 g). The mixture was washed with small aliquots (5 × 1 mL) of MeOH. The solid residue, soluble in $CHCl_3$−MeOH (1:1), essentially contained cytochalasin B (**1**), as shown by TLC analysis carried out in comparison with an authentic sample of the toxin [R_f 0.71, using as solvent system $CHCl_3$−*i*-PrOH (9:1) and R_f 0.47 using EtOAc−*n*-hexane (7:3)]. The solid was crystallized twice by EtOAc−*n*-hexane (1:5), giving white needles (5.3 g, 2.12 g/kg dry weight) of cytochalasin B (**1**). The mother liquors of cytochalasin B crystallization were combined with the initial methanol fraction (a total of 1.7 g) and fractionated by medium-pressure (15 bar) column chromatography eluted with $CHCl_3$−*i*-PrOH (9:1), yielding eight groups of homogeneous fractions. The groups 2−5 contained conspicuous amounts of cytochalasins B, as well as of other known cytochalasins, i.e., A, F, T, 19-*O*-acetylcytochalasin B, and deoxaphomin, which were purified as previously reported.[5] The residue of the seventh fraction group (53.2 mg) was further purified by preparative TLC (eluent EtOAc−*n*-hexane, 7:3), giving three fractions (R_f 0.32, 5.0 mg; R_f 0.25, 19.3 mg; and R_f 0.17, 10.2 mg, respectively). The most polar fraction consisted of a pure amorphous solid, which proved to be cytochalasin Z3 (**5**), by comparison with an authentic sample.[1] The fraction with intermediate polarity was further purified by the same method, but using $CHCl_3$−*i*-PrOH (9:1) as eluent system, yielding three more fractions (R_f 0.56, 3.5 mg; R_f 0.50, 11.0 mg; and R_f 0.44, 3.3 mg, respectively). The first, appearing as a homogeneous amorphous solid (R_f 0.60, by TLC on reversed-phase, eluent EtOH−H_2O, 6:4) was cytochalasin Z4 (**6**) and the second was identified as cytochalasin Z2 (**4**),[1] by comparison with an authentic sample. The third fraction (3.3 mg) was further purified by two successive steps of preparative TLC on reversed-phase and silica gel plates (eluent EtOH−H_2O, 6:4, and $CHCl_3$-*i*-PrOH, 97:3), yielding a small amount (0.5 mg) of deoxaphomin[15] and of an amorphous homogeneous solid (0.2 mg, R_f 0.58 and 0.48 in the two above-described TLC systems), which proved to be cytochalasin Z6 (**8**). Five fractions were obtained fractioning the residue (47.3 mg) of the initial eighth fraction, by preparative TLC (eluent $CHCl_3$−*i*-PrOH, 97:3). Among these, the less polar (R_f 0.38, 2.3 mg) was purified by the same procedure but using EtOAc−*n*-hexane (7:3) as eluent, yielding a pure amorphous solid (0.7 mg, R_f 0.28 and 0.54 by

TLC on reversed-phase, eluent EtOH−H_2O, 6:4), which afforded cytochalasin Z5 (**7**).

Cytochalasin Z4 (6):[16] UV λ_{max} (log ϵ) 276 (3.13) nm; IR ν_{max} 3356, 1708, 1653, 1599, 1500, 1457, 1264, 1217 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HMBC δ 171.7 (C-1): 3.29 (H-8), 164.6 (C-23): 7.01 (H-21) and 5.86 (H-22), 154.4 (C-2′): 7.09 (H-4′), 7.05 (H-6′), 2.94 (H-10) and 2.82 (H-10′), 152.2 (C-21): 1.88 (H-19), 148.4 (C-6): 5.39 (H-12), 5.16 (H-12′), 3.89 (H-7), 3.29 (H-8) and 1.12 (Me-11), 138.1 (C-14): 3.29 (H-8) and 1.72 (H-15′), 131.5 (C-6′): 7.09 (H-4′) and 6.83 (H-5′), 128.4 (C-4′): 7.05 (H-6′) and 6.83 (H-5′), 126.3 (C-13): 3.29 (H-8) and 1.72 (H-15′), 123.8 (C-1′): 2.94 (H-10) and 2.82 (H-10′), 120.7 (C-5′): 7.05 (H-6′) and 6.81 (H-3′), 119.3 (C-22): 7.01 (H-21), 115.8 (C-3′): 6.83 (H-5′), 83.1 (C-9): 6.20 (NH) and 3.29 (H-8), 70.9 (C-20): 7.01 (H-21), 5.86 (H-22), 1.88 (H-19) and 1.44 (H-18), 68.9 (C-7): 5.39 (H-12), 5.16 (H-12′) and 3.29 (H-8), 53.3 (C-3): 6.20 (NH), 2.94 (H-10) and 2.82 (H-10′), 49.5 (C-4): 6.20 (NH), 2.94 (H-10) and 2.82 (H-10′), 49.4 (C-8): 5.92 (H-13), 41.7 (C-15): 5.92 (H-13) and 0.88 (Me-C-16), 38.6 (C-10): 7.05 (H-6′), 35.0: (C-17): 1.88 (H-19), 1.72 (H-15′) and 0.88 (Me-C-16), 34.8 (C-19): 1.65 (H-17), 33.0 (C-5): 5.39 (H-12), 5.16 (H-12′) and 1.12 (Me-11), 20.2 (C-18): 1.88 (H-19), 1.65 (H-17), 1.60 (H-19′) and 0.65 (H-17′); EIMS *m/z* (rel int) 495 $[M]^+$ (19), 477 $[M - H_2O]^+$ (9), 459 $[M - 2\times H_2O]^+$ (2.5), 388 $[M - C_7H_6OH]^+$ (63), 370 $[M - H_2O - C_7H_6OH]^+$ (63), 352 $[M - 2\times H_2O - C_7H_6OH]^+$ (32), 107 $[C_7H_6OH]^+$ (100); ES MS (+) *m/z* 534 $[M + K]^+$, 518 $[M + Na]^+$, 496 $[M + H]^+$, 389 $[M + H - C_7H_6OH]^+$; ES MS (−) *m/z* 494 $[M - H]^-$, 476 $[M - H - H_2O]^-$, 458 $[M - H - 2\times H_2O]^-$; HREIMS *m/z* 495.2610 $[M]^+$ (calcd for $C_{29}H_{37}NO_6$, 495.26229).

Cytochalasin Z5 (7):[16] UV λ_{max} (log ϵ) 278 (3.02) nm; IR ν_{max} 3398, 1709, 1651, 1614, 1521, 1475, 1264 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HMBC δ 171.1 (C-1): 3.31 (H-8), 164.4 (C-23): 7.01 (H-21) and 5.87 (H-22), 154.5 (C-4′): 7.02 (H-2′ and/or H-6′), 6.76 (H-3′ and/or H-5′), 152.7 (C-21): 1.93 (H-19), 148.2 (C-6): 1.11 (Me-11), 138.3 (C-14): 3.31 (H-8) and 1.74 (H-15′), 129.1 (C-1′): 6.76 (H-3′ and/or H-5′), 2.78 (H-10) and 2.71 (H-10′), 126.3 (C-13): 3.31 (H-8) and 1.74 (H-15′), 115.8 (C-3′, 5′): 7.02 (H-2′ and/or H-6′), 82.7 (C-9): 5.61 (NH), 3.31 (H-8), 70.9 (C-20): 7.01 (H-21), 5.87 (H-22) and 1.93 (H-19), 69.3 (C-7): 5.39 (H-12), 5.17 (H-12′) and 3.31 (H-8), 53.4 (C-3): 5.61 (NH), 2.78 (H-10) and 2.71 (H-10′), 49.5 (C-8): 5.45 (H-14), 49.2 (C-4): 5.61 (NH) and 1.11 (Me-11), 43.2 (C-10): 7.02 (H-2′ and/or H-6′), 41.8 (C-15): 5.93 (H-13) and 0.89 (Me-C-16), 35.0: (C-17): 2.12 (H-15), 1.93 (H-19) and 0.89 (Me-C-16), 32.8 (C-16): 2.12 (H-15), 1.74 (H-15′) 0.89 (Me-C-16), 31.8 (C-5): 5.39 (H-12), 5.17 (H-12′) and 1.11 (Me-11), 20.2 (C-18): 0.65 (H-17′); EIMS *m/z* (rel int) 495 $[M]^+$ (0.6), 477 $[M - H_2O]^+$ (0.8), 448 $[M - H_2O - HCO]^+$ (0.4), 433 $[M - H_2O - HCO - Me]^+$ (0.6), 388 $[M - C_7H_6OH]^+$ (6), 370 $[M - H_2O - C_7H_6OH]^+$ (7), 352 $[M - 2\times H_2O - C_7H_6OH]^+$ (4), 107 $[C_7H_6OH]^+$ (100); ES MS (+) *m/z* 534 $[M + K]^+$, 518 $[M + Na]^+$, 496 $[M + H]^+$; HREIMS *m/z* 495.2612 $[M]^+$ (calcd for $C_{29}H_{37}NO_6$, 495.2622).

Cytochalasin Z6 (8):[16] UV λ_{max} <220 nm; IR ν_{max} 3467, 1710, 1652, 1606, 1467, 1263 cm^1; ^1H and ^{13}C NMR, see Table 1; HMBC δ 171.1 (C-1): 3.06 (H-8), 164.4 (C-23): 5.72 (H-22), 146.7 (C-21): 5.72 (H-22) and 3.74 (H-19), 136.9 (C-1′): 7.33 (H-3′ and/or H-5′), 129.1 (C-2′,6′): 7.25 (H-4′) and 7.33 (H-3′ and/or H-5′), 128.9 (C-3′, 5′): 7.15 (H-2′ and/or H-6′), 127.0 (C-4′): 7.15 (H-2′ and/or H-6′), 126.5 (C-13): 2.73 (H-7), 71.5 (C-19): 2.81 (H-20) and 2.21 (H-20′), 61.0 (C-7): 1.22 (Me-12), 57.3 (C-6): 2.32 (H-5), 1.22 (Me-12) and 1.04 (Me-11), 49.3 (C-4): 1.04 (Me-11), 41.5 (C-15): 0.88 (Me-C-16), 35.8 (C-5): 1.22 (Me-12) and 1.04 (Me-11), 33. (C-16): 1.28 (H-18′), 31.1 (C-17); 1.32 (H-16); EIMS *m/z* (rel int) 479 $[M]^+$ (1), 464 $[M - Me]^+$ (4), 461 $[M - H_2O]^+$ (1.6), 446 $[M - Me - H_2O]^+$ (1), 428 $[M - Me - 2\times H_2O]^+$ (2), 400 $[M - 2\times H_2O - Me - CO]^+$ (1), 389 $[M + H - C_7H_7]^+$ (1), 388 $[M - C_7H_7]^+$ (7), 370 $[M - C_7H_7 - H_2O]^+$ (8), 352 $[M - C_7H_7 - 2\times H_2O]^+$ (0.5), 91 $[C_7H_7]^+$ (100); ES MS (+) *m/z* 502 $[M + Na]^+$, 480 $[M + H]^+$, 462 $[M + H - H_2O]^+$; HREIMS *m/z* 479.2662 $[M]^+$ (calcd for $C_{29}H_{37}NO_5$, 479.2673).

Bioassay Methods. Phytotoxicity of the new cytochalsins Z4, Z5, and Z6 was determined using an assay on germinating tomato seedlings as previously described,[1] whereas the zootoxicity was evaluated using a brine shrimp assay.[17]

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