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Phomachalasins A–D, 26-oxa[16] and [15]cytochalasans produced by *Phoma exigua var. exigua*, a potential mycoherbicide for *Cirsium arvense* biocontrol

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ABSTRACT

Phoma exigua var. exigua, a fungal pathogen isolated from *Cirsium arvense* and *Sonchus arvensis*, proposed as a biocontrol agent of this noxious perennial weeds, produces in liquid and solid cultures different phytotoxic metabolites with potential herbicidal activity. The phytotoxic cytochalasins B, F, Z2, and Z3 and deoxaphomin were previously identified together with *p*-hydroxybenzaldehyde. Using spectroscopic methods, four new cytochalasins, termed phomachalasins A–D, were isolated and characterized as three new closely related 26-oxa[16] and one new [15]cytochalasans. They belong to a new subgroup of cytochalasans bearing a 1,2,3,4,6,7-hexasubstituted bicycle[3.2.0]heptene joined to the macrocyclic ring. None of the four new metabolites showed phytotoxic or antimicrobial activity. The lack of both phytotoxic and antimicrobial activities showed by all phomachalasins A–D was probably due to the strong modification of both functionalities and conformational freedom of the macrocyclic ring caused by its junction with the bulky and quite rigid new bicycle, namely bicycle[3.2.0]heptene.

1. Introduction

Perennial weeds give rise to widespread problems in crop production. They are especially harmful in agricultural systems with reduced herbicide usage due to the ineffectiveness of mechanical weed control. Typical weed species include the Asteraceae *Cirsium arvense* (L.) Scop. and *Sonchus arvensis* (L.), commonly called Canada thistle and perennial sowthistle, respectively. The few herbicides recommended for chemical control of these perennials in non-organic cropping systems have low selectivity. Microbial phytotoxins or their synthetic analogues may be used to develop new agrochemicals against such weeds. Several pathogens, namely *Stagonospora cirsii* J.J. Davis, *Ascochyta sonchi* (Sacc.) Grove and related pathogens, were found to be common on both host plants, and to produce phytotoxic metabolites. *Phyllosticta cirsii* Desm. and *Phomopsis cirsii* Grove, belonging to well-known genera for toxin production, were also proposed for biocontrol of *C. arvense*, while *Alternaria sonchi* was recently proposed as a mycoherbicide for the control of *S. arvensis*. Several new phytotoxins, belonging to different groups of natural compounds, were isolated as potential herbicides from these fungi. These comprise the nine stagonolides and modiolide A isolated from *S. cirsii*,[1,2] ascosonchine isolated from *Ascochyta sonchi*,[3] phyllostictines A–D,[4] phyllostoxin and phyllostin isolated from *Phyllosticta cirsii*,[5] 3-nitropropanoic acid isolated from *Phomopsis cirsii*[6] and alternethanoxins A and B isolated from *Alternaria sonchi*.[7]

Several fungi were previously isolated from diseased leaves of *C. arvense* L. and *S. arvensis* L. and preliminarily identified as *Ascochyta sonchi* (Sacc.) Grove according to the *Ascochyta* manual.[8] Other taxonomic studies reclassified *A. sonchi* as a component of the complex species *Phoma exigua* Desm. var. *exigua* but thistles were not mentioned as hosts.[9,10] Their potential for the biological control of such perennial weeds, which widely occur through temperate regions of the world,[11,12] was evaluated.[13,14] Most of the above strains produced ascosonchine, whereas strains C-177 and S-9 grown in liquid and solid cultures, though virulent to weeds, did not produce the above metabolite, but produced different toxic metabolites identified as cytochalasins B, F, Z2, and Z3 and deoxaphomin, and *p*-hydroxybenzaldehyde. When assayed on leaves of both *C. arvense* and *S. arvensis*, *p*-hydroxybenzaldehyde was inactive, whereas

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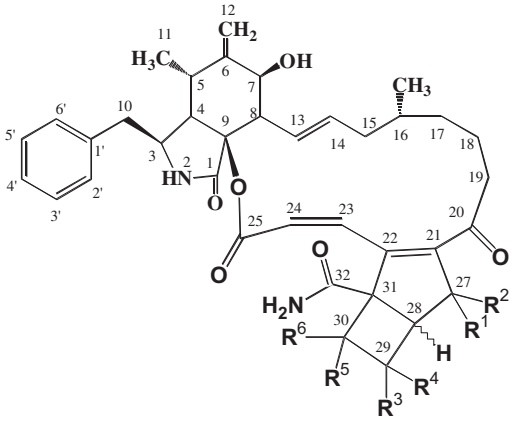
deoxaphomin demonstrated the highest level of toxicity on leaves of *S. arvensis*. Cytochalasin Z2 appeared to be the least toxic cytochalasan on both plants according to the lack of the secondary hydroxyl group on C-7. Production of cytochalasins by *P. exigua* var. *exigua* strains isolated from *C. arvense* and *S. arvensis* was discussed in relation with chemotaxonomy and biocontrol potential of the fungus.[15]

This paper describes the isolation, structural elucidation, and biological characterization of four new cytochalasins produced both in liquid and solid culture by *P. exigua var. exigua* (strain C-177), termed phomachalasins A–D (**1**–**4**). Their structures were determined by the extensive use of NMR and MS techniques.

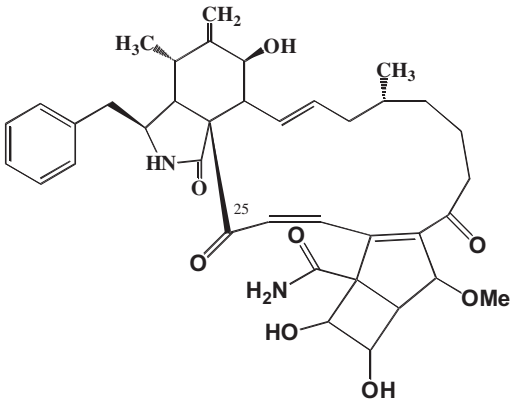
2. Results and discussion

The solid culture of *Phoma exigua var. exigua* (800 g) was exhaustively extracted and the highly phytotoxic organic extract was purified by a combination of CC and TLC (see Experimental section). In addition to the already reported cytochalasins B (**5**) and F, deoxaphomin, and *p*-hydroxybenzaldehyde, we obtained four metabolites, one as crystalline and three as amorphous solids (25.1, 3.1, and 3.3 mg/kg, respectively), which we called phomachalasins A–C (**1**–**3**, Fig. 1). Purification of the organic extract obtained from a liquid culture of the same fungus, together with *p*-hydroxybenzaldehyde and cytochalasin B, yielded a further amount of phomachalasin B (1.2 mg/L) and an amorphous solid metabolite named phomachalasin D (**4**, 0.8 mg/L). Preliminary ^{1}H and ^{13}C spectra, compared to those of cytochalasin B recorded in the same solvent,[16,17] indicated that the structures of all metabolites are closely related, and constitute four novel cytochalasins belonging to a new subgroup of cytochalasans. Indeed, **1**, **3**, and **4**, compared to cytochalasin B,[16,17] showed both unaltered benzyl and perhydroisoindolyl residues, which are joined to a similar macrocyclic ring. The same relation exists between phomachalasin B (**2**) and deoxaphomin,[18] a deoxa [13]cytochalasan closely related to **5**. Furthermore, the ^{1}H and ^{13}C NMR spectra of all four novel cytochalasins present signals originating from a carbonyl group at C-20 as in cytochalasin A,[16] and from a 6,7-dihydroxy-4-methoxy-bicyclo[3.2.0]hept-2-ene-1-carboxylic acid amide, with different stereochemistry at its chiral centres as detailed below, inserted between the C-20 and the C-23 (C-21 in cytochalasin A) of the macrocyclic ring.

Phomachalasin A (**1**) is the main metabolite, obtained as a crystalline compound. Unfortunately the crystals as well as the others obtained from slow evaporation of pure and mixed solvents, appeared unsuitable for X-ray analysis. As inferred from HRESIMS spectra, phomachalasin A has the molecular formula $C_{38}H_{46}N_2O_9$, consistent with 17° of unsaturation. 13 of them are due to the benzyl and perhydroisoindolyl residues and macrocyclic ring as in cytochalasin A. The remaining four were due to the 1,2,3,4,6,7-hexasubstituted bicycloheptene moiety. The IR spectrum showed bands attributable to hydroxyl, amide, carbonyl, olefinic, and aromatic groups.[19] The UV spectrum exhibited, with respect to cytochalasin B, a maximum absorption typical of an extended conjugated α,β-unsaturated lactone group.[20] The ^{1}H NMR spectrum (Table 1), compared with those of cytochalasins A and B recorded in the same conditions, overlap, except for the proton signals (H-23 and H-24) of the double bond α,β-located with respect to the lactone of the macrocyclic ring. As inferred from the typical coupling value[21] it assumes a *cis*-instead of a *trans*-configuration. Further significant differences were due to the signal systems of four secondary carbons, three of which are oxygenated, whose methine protons couplings appeared in the COSY spectrum.[22] In fact, the proton H-27 resonating at δ 5.13 as a doublet (J=8.3 Hz) coupled with H-28 resonating at δ 3.77 as a double doublet (J=8.3 and 4.5 Hz). The latter coupled with H-29, which is another double doublet (J=4.7 and 4.5 Hz) observed at δ 4.29, and coupled in turn



1 $R^1 = OCH_3$, $R^2 = R^3 = R^5 = H$, $R^4 = R^6 = OH$
3 $R^1 = OCH_3$, $R^2 = R^4 = R^6 = H$, $R^3 = R^5 = OH$
4 $R^1 = OCH_3$, $R^2 = R^3 = R^6 = H$, $R^4 = R^5 = OH$



2

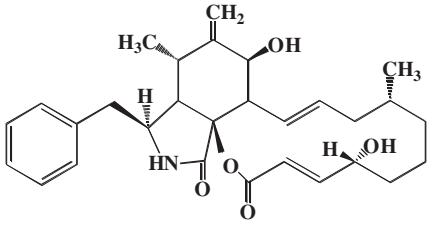


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Fig. 1. Structures of phomachalasins A–D (**1**–**4**).

Table 1
^{1}H NMR data of phomachalasins A–D (**1**–**4**)[a,b]

Compound	**1**	**2**	**3**	**4**
Position	δ_H	δ_H	δ_H	δ_H
3	3.18–3.16m	3.37–3.34m	3.36–3.33m	3.33–3.31m
4	2.19 dd (*J*=4.6, 4.2 Hz)	2.38 dd (*J*=4.1, 4.0 Hz)	2.79 dd (*J*=4.0, 3.8 Hz)	2.70 dd (*J*=4.2, 3.6 Hz)
5	2.83–2.87m	3.10–2.95m	3.07–3.01m	3.53–3.48m
7	3.74 d (*J*=12.1 Hz)	4.00 d (*J*=11.3 Hz)	3.79 d (*J*=11.6 Hz)	3.79 d (*J*=10.9 Hz)
8	2.58 dd (*J*=12.1, 9.4 Hz)	3.19 dd (*J*=11.3, 9.8 Hz)	2.81 dd (*J*=11.6, 9.5 Hz)	3.36 dd (*J*=10.9, 9.8 Hz)
10	2.92 dd (*J*=13.4, 10.7 Hz)	2.87 dd (*J*=13.4, 4.1 Hz)	3.16 dd (*J*=13.5, 11.1 Hz)	2.91 dd (*J*=13.6, 3.5 Hz)
	2.77–2.71m	2.80 dd (*J*=13.4, 10.4 Hz)	2.90 dd (*J*=13.5, 2.9 Hz)	2.67 dd (*J*=13.6, 9.4 Hz)
11	1.09 d (*J*=6.6 Hz)	1.12 d (*J*=6.7 Hz)	0.88 d (*J*=6.6 Hz)	1.18 d (*J*=6.6 Hz)
12	5.43s	5.45s	5.53s	5.42s
	5.15s	5.20s	5.23s	5.21s
13	5.54 dd (*J*=15.3, 9.4 Hz)	5.72 dd (*J*=15.2, 9.8 Hz)	5.72 dd (*J*=15.2, 9.5 Hz)	5.79 dd (*J*=15.1, 9.8 Hz)
14	5.45 ddd (*J*=15.3, 10.4, 3.9 Hz)	5.50 ddd (*J*=15.2, 11.0, 3.6 Hz)	5.48 ddd (*J*=15.2, 10.6, 3.3 Hz)	5.29 ddd (*J*=15.1, 12.1, 4.3 Hz)
15	2.13 br dd (*J*=12.9, 3.9 Hz)	2.12–1.96m	2.18 br d (*J*=14.9, 3.3 Hz)	1.97–1.90m
	1.79–1.70m	1.72 ddd (*J*=14.6, 14.6, 11.0 Hz)	1.79 ddd (*J*=14.9, 14.9, 10.6 Hz)	1.70–1.66m
16	1.57–1.52m	1.28–1.22m	1.54–1.50m	1.14–1.11m
17	1.57–1.52m	1.03–0.87 (2H)m	1.43–1.38m	1.14–1.11m
	1.30–1.25m		0.84–0.81m	0.71–0.66m
18	1.79–1.70 (2H)m	1.33–1.28m	1.67–1.62m	1.70–1.66m
		1.23–1.21m	1.54–1.50m	1.32–1.25m
19	2.77–2.71m	2.12–1.96m	2.47–2.43m	2.11–2.07m
	2.44–2.42m	1.95–1.92m	2.23–2.20m	2.05–2.00m
23	7.27 d (*J*=8.4 Hz)	7.32 d (*J*=8.4 Hz)	7.41 d (*J*=8.5 Hz)	7.35 d (*J*=8.3 Hz)
24	6.85 d (*J*=8.4 Hz)	6.84 d (*J*=8.4 Hz)	6.84 d (*J*=8.5 Hz)	6.90 d (*J*=8.3 Hz)
27	5.13 d (*J*=8.3 Hz)	5.58 d (*J*=6.7 Hz)	5.62 d (*J*=4.1 Hz)	5.29 d (*J*=5.8 Hz)
28	3.77 dd (*J*=8.3, 4.5 Hz)	3.50 dd (*J*=6.7, 2.9 Hz)	4.01 br d (*J*=4.1 Hz)	2.85 d (*J*=5.8 Hz)
29	4.29 dd (*J*=4.7, 4.5 Hz)	4.26 dd (*J*=10.0, 2.9 Hz)	4.59 br d (*J*=7.5 Hz)	3.98 d (*J*=10.1 Hz)
30	4.97 d (*J*=4.7 Hz)	4.36 d (*J*=10.0 Hz)	3.89 d (*J*=7.5 Hz)	4.56 d (*J*=10.1 Hz)
Me-16	0.92 d (*J*=6.6 Hz)	0.83 d (*J*=6.8 Hz)	1.23 d (*J*=6.6 Hz)	0.68 d (*J*=6.5 Hz)
2′,6′	7.10 d (*J*=7.3 Hz)	7.19 d (*J*=7.3 Hz)	7.21 d (*J*=7.5 Hz)	7.16 d (*J*=7.4 Hz)
3′,5′	7.28 d (*J*=7.3 Hz)	7.33 d (*J*=7.3 Hz)	7.31 d (*J*=7.5 Hz)	7.36 d (*J*=7.4 Hz)
4′	7.23 dd (*J*=7.3, 7.3 Hz)	7.27 dd (*J*=7.3, 7.3 Hz)	7.25 dd (*J*=7.5, 7.5 Hz)	7.28 ddd (*J*=7.4, 7.4 Hz)
NH	5.51s	5.61s	5.67s	5.54s
NH_2	6.41 br s	6.41 br s	6.41 br s	5.17 br s
OMe	3.61s	3.73s	3.45s	3.64s

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

with H-30, a doublet (*J*=4.7 Hz) at δ 4.97. Both carbons C-29 and C-30 bear a hydroxy group while a methoxy group, appearing as a singlet at δ 3.61, was located on carbon C-27 on the basis of the coupling between OMe and H-27 observed in the NOESY spectrum[22] of **1** (Table 4). H-28 was bonded to one of the headbridge carbons (C-28) of the junction between the cyclobutane and cyclopentene rings of the 1,2,3,4,6,7-hexasubstituted bicycloheptene moiety, with the other (C-31) bearing a carbamide group. The primary amide also generated a typical band in the IR spectrum, and in the ^{1}H NMR spectrum appeared as a very broad singlet at δ 6.41.[21] These identifications were corroborated by the signal pattern observed in the ^{13}C NMR spectrum (Table 2) that differed from those of the cytochalasins A and B[16,17] only in the signals of the 1,2,3,4,6,7-hexasubstituted bicyloheptene moiety, which were attributed on the basis of the couplings observed in the HSQC and HMBC spectra[22] of **1**. The corresponding amidic carbonyl, the three oxygenated secondary carbons, and the secondary and quaternary carbons appeared at δ 170.5, 85.2, 80.2 and 71.9, 46.7, and 57.5 (C-32, C-27, C-29 and C-30, C-28, and C-31), respectively, while the two quaternary olefinic carbons (C-22 and C-21) and the methoxy group resonated at the typical chemical shift values of δ 155.8, 131.5, and δ 58.9, respectively.[23] These findings allowed assignment of all the protons and the corresponding carbons, whose chemical shifts are reported in Tables 1 and 2, respectively.

Furthermore, phomachalasin A corresponds to structure **1**, a new 26-oxa[16]cytochalasan. This structure was supported by several couplings identified in the HMBC spectrum (Table 3). We also observed significant couplings between the macrocyclic ring and the 1,2,3,4,6,7-hexasubstituted bicycloheptene moiety, and those involving the different fragments of the latter. We detected the coupling between H-28 and C-20, between both H-24 and H-30 with C-21, both H-23 and H-24 with C-22, H-30 with C-23, H-28 with C-27, both H-27 and H-30 with C-28, H-30 with C-29, both H-23 and H-28 with C-30, and finally both H-27 and H-29 with C-32. The structure was further confirmed by the sodium clusters observed in the HRESIMS spectrum for the toxin itself and its dimmer at *m*/*z* 697.3099 $[M+Na]^+$ and 1371 $[2M+Na]^+$, respectively. The relative stereochemistry of the chiral carbons in the perhydroinsoindolyl residues and macrocyclic ring are assigned by comparing the ^{1}H NMR data of **1** with those of cytochalasin B, and was confirmed by the couplings observed in the NOESY spectrum (Table 4) for these moieties. The couplings observed in the ^{1}H NMR and NOESY spectra also allowed the assignment of the relative stereochemistry with respect to the chiral carbons of the 1,2,3,4,6,7-hexasubstituted bicycloheptene moiety, which is reported in the structural formulas of **1**. On these findings both H-27 and H-28 and H-29 and H-30 appeared to be *cis* while H-28 and H-29 appeared to be *trans*.

The other three phomachalasins B–D (**2**–**4**) appear to be very closely related to phomachalasin A. Phomachalasin B (**2**), obtained from solid and liquid culture, has a molecular formula $C_{38}H_{46}N_2O_8$, as inferred from HRESIMS spectra again consistent with the same 17° of unsaturation as in **1**, which are in agreement with the IR bands, UV absorption, and ^{1}H and ^{13}C NMR investigations. Therefore, **2** differed from **1** in the absence of an oxygen atom. ^{1}H and ^{13}C NMR spectra (Tables 1 and 2) indicated that this difference is due to the lack of the lactone functionalities closing the macrocyclic ring that assumes a carbocyclic nature as in deoxaphomin. Indeed, this carbonyl group (C-25) appeared significantly downfield shifted ($\Delta\delta$ 36.5) at δ 206.5 as previously observed comparing deoxaphomin to

Table 2
^{13}C NMR data of phomachalasins A–D (**1**–**4**)[a,b]

Compound	**1**	**2**	**3**	**4**
Position	δ_C m[c]	δ_C m[c]	δ_C m[c]	δ_C m[c]
1	170.5 s	173.0 s	171.2 s	169.5 s
3	54.1 d	54.4 d	54.6 d	53.3 d
4	48.9 d	49.3 d	49.3 ds	49.4 ds
5	32.5 d	32.6 d	32.0 d	32.2 d
6	148.4 s	148.2 s	148.2 s	148.5
7	67.1 d	69.4 d	66.5 d	69.31
8	50.1 d	49.4 d	50.5 d	48.2 d
9	83.3 s	62.116	84.1 s	84.6 s
10	43.0 t	44.5 t	43.1 ts	44.5 t
11	15.3 q	14.4 q	15.7 q	14.4 q
12	113.7 t	114.7 t	113.8 t	106.1 t
13	126.0 d	126.2 d	125.4 d	127.2 d
14	137.9 d	138.5 d	138.7 d	136.5 d
15	40.4 t	41.8 t	40.4 t	41.4 t
16	31.0 d	32.4 d	31.6 d	31.6 d
17	31.8 t	36.3 t	32.5 t	31.9 t
18	20.5 t	23.2 t	22.0 t	23.1 t
19	39.9 t	42.3 t	39.6 t	37.7 t
20	204.3 s	203.7 s	206.6 s	204.5 s
21	131.5 s	130.3 s	133.7 s	129.9 s
22	155.8 s	156.0 s	155.2 s	156.6 s
23	128.9 d	129.1 d	129.0 d	129.2 d
24	115.4 d	115.6 d	115.1 d	115.9 d
25	170.0 s	206.5 s	170.9 s	169.5 s
27	85.2 d	82.9 d	75.0 d	76.1 d
28	46.7 d	54.7 d	59.5 d	60.3 d
29	80.2 d	80.5 d	70.5	79.5 d
30	71.9 d	73.9 d	80.0 d	73.9 d
31	57.5 s	57.3 s	58.0 s	56.5 s
32	170.5 s	173.0 s	171.2	169.5 s
Me-16	20.3 q	22.5 q	19.6 q	20.4 q
1′	137.8 s	137.4 s	137.6 s	136.8 s
2′,6′	129.0 d	129.4 d	129.1 d	129.1 d
3′,5′	127.6 d	129.0 d	128.0 d	130.1 d
4′	127.0 d	127.2 d	127.2	127.3 d
OMe	58.9 q	58.9 q	58.3 q	58.8 q

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

cytochalasin B.[17,18] On the basis of the couplings observed in the COSY and HSQC spectra the proton and corresponding carbon chemical shifts were assigned (Tables 1 and 2) and the structure **2** of a novel [15]cytochalasan was attributed to phomachalasin B. Such a structure was supported by the couplings observed in the HMBC spectrum (Table 3), and in particular, by that observed between NH and C-25. The structure was further confirmed by the potassium and sodium clusters observed in the HRESIMS spectrum at *m*/*z* 697 $[M+K]^+$ and 681.3150 $[M+Na]^+$, respectively. The relative stereochemistry of the chiral carbons in the 1,2,3,4,6,7-hexasubstituted bicycloheptene was identical to that of **1** as derived on the basis of the couplings observed in the ^1H NMR and NOESY spectra of phomachalasin B.

Phomachalasins C and D (**3** and **4**), obtained, respectively, from solid and liquid culture, both have the same molecular formula ($C_{38}H_{46}N_2O_9$), as deduced from the HRESIMS spectrum, and consistent with the same 17° of unsaturation found in **1**. On comparing the ^1H and ^{13}C NMR spectra of phomachalasins C and D with those of **1**, we noticed that the two compounds differed from phomachalasin A in the relative stereochemistry of chiral carbons of the 1,2,3,4,6,7-hexasubstitute bicycloheptene moiety. On the basis of the coupling observed in the ^1H NMR and NOESY spectra compared to **1**, we observed in **3** an inverted *cis*-stereochemistry between H-28 and H-29, and in **4** an inverted *trans*-stereochemistry between H-29 and H-30. The couplings observed in the COSY and HSQC spectra allowed the assignment to all the protons and corresponding carbons (Tables 1 and 2), and structures **3** and **4** to phomachalasins C and D, two novel 26-oxa[16]cytochalasans, that are diastereomers of phomachalasin A. These structures were confirmed by several couplings observed in the HSQC and NOESY spectra (Tables 3 and 4) as reported above for **1** and **2**. Further support was obtained from their HRESIMS spectra, which showed for both cytochalasins the sodium cluster $[M+Na]^+$ at *m*/*z* 697.3103 and 697.3098, respectively.

When tested at the concentration 2 mg/mL on leaf segments of three different plant species, none of the metabolites showed any phytotoxic activity. Nor did compounds **1**–**4** possess antimicrobial activity when tested at 100 μg/disk.

The lack of phytotoxic and antimicrobial activity shown by all phomachalasins A–D is probably due to the strong modification of both the functionalities and conformational freedom of the macrocyclic ring induced by the junction of the latter with the bulky and quite rigid 1,2,3,4,6,7-hexasubstituted bicycle[3.2.0]heptene, as also observed upon inspection of a Drieding model. This result came as no surprise since both functionalities and conformational freedom of the macrocyclic ring appeared to be, in previously structure–activity relationship studies carried out among the cytochalasin group,[15,24–28] important structural features to impart both phytotoxic and antimicrobial activity.

3. Conclusion

Although the four phomachalasins belong to the well-known family of cytochalasins, fungal metabolites also known for their original chemical and interesting biological activities,[24–26,29,30] they are the first four fungal metabolites belonging to a new subgroups of cytochalasans. The main structural novelty is the 1,2,3,4,6,7-hexasubstituted bicycloheptene with different stereochemistry at its chiral carbons, inserted between the C-20 and C-23 of the macrocyclic ring. However, phomachalasins A–D lose biological activity compared to well-known cytochalasins such as cytochalasin B and deoxaphomin.[28]

4. Experimental section

4.1. General

Optical rotations were measured in $CHCl_3$ solution on a Jasco P-1010 digital polarimeter; IR spectra were recorded as a glassy film on a Perkin–Elmer Spectrum One FT-IR spectrometer and UV spectra were taken in MeCN solution on a Perkin–Elmer Lambda 25 UV/vis spectrophotometer. ^1H and ^{13}C NMR spectra were recorded at 600 and at 150 MHz, respectively, in $CDCl_3$ on Bruker spectrometer. The solvent was used as internal standard. Carbon multiplicities were determined by DEPT spectra.[22] DEPT, COSY-45, HSQC, HMBC, and NOESY experiments[22] were performed using Bruker microprograms. ESI and HRESI MS spectra were recorded on Waters Micromass Q-TOF Micro and Agilent 1100 coupled to a JOEL AccuTOF (JMS-T100LC) spectrometer. 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, 0.063–0.200 mm). Solvent systems: (A) $CHCl_3$–*i*-PrOH (98:2); (B) EtOAc–*n*-hexane (6:4); (C) petroleum ether–Me_2CO; (65:35) (D) EtOH–H_20 (6:4).

4.2. Fungus

Fungus was isolated from necrotic lesions on leaves of *C. arvense* and *S. arvensis*. They were collected in St. Petersburg and

Table 3
HMBC data of phomachalasins A–D (**1**–**4**)

C	**1**	**2**	**3**	**4**
	HMBC	HMBC	HMBC	HMBC
1	H-7, H-8, NH	H-8, NH	H-8	H-8
3	H-4, H-8, H_2-10, NH	H-10, H-10′, NH	H-10, H-10′, NH	H-4, H-10, H-10′, NH
4	H-5, H_3-11	H_3-11, NH	H-10, H-10′, H_3-11, NH	H_3-11, NH
5	H-3, H_2-12	H-4, H_3-11, H-12, H-12′	H-3, H_3-11	H_3-11, H-12, H-12′
6	H-4, H-7, H-8, H_2-12,H_3-11	H-4, H-7, H_3-11, H-12, H-12′	H-4, H-7, H_3-11, H-12, H-12′	H-4, H-7, H_3-11, H-12
7	H-8, H_2-12		H-8, H-12, H-12′	H-8, H-12, H-12′
8	H-7, H-13	H-3, H-5, H-14, NH	H-7, H-13, H-14	H-13
9	H-4, H-7, H-8, NH	H-3, NH	H-4, H-8, H	H-8, NH, H-4
10	H-4	H-4, H-5, H-3′,5′	H-3, H-4, H-5, H-2′,6′	
11				
12	H-7	H-7	H-7,	H-7
13	H-7, H-8, H-15, H-15′	H-8, H-15, H-15′	H-7, H-8, H-15, H-15′	H-8, H-15, H-15′
14	H-8, H_2-12, H-13, H-15, H-15′	H-8, H-15′	H-8, H-15, H-15′	H-8, H-15, H-15′
15	H-6, Me-16	H-13, Me-16		Me-16
16	H-15, H-15′, Me-16	H-15, H-15′, H-18, Me-16	H-13, H-14, H-15, H-15′, H-17, H-17′	H-15, H-15′, H-17, Me-16
17	H-19, H-19′	H-19, Me-16	H-19, H-19′, Me-16	H-18
18	H-17, H-17′, H-19, H-19′	H-19, H-19′	H-17, H-17′, H-19, H-19′	
19	H_2-18		H-18, H-18′	
20	H-19, H-19′, H_2-18, H-28	H-19, H-19′,H-18, H-18′, H-28		H-27, H-28,
21	H-24, H-30	H-24	H-24, H-29, H-30	
22	H-23, H-24	H-23, H-24	H-23, H-24	H-23, H-24
23	H-30	H-30	H-24	H-24, H-30
24	H-23	H-23	H-23	
25		NH		
27	H-28	H-28		H-28, H-29
28	H-27, H-30	H-27	H-27, H-29	
29	H-30	H-30	H-28, H-30	H-30
30	H-23, H-28	H-23, H-28, H-29	H-28, H-29	
31				
32	H-27, H-29		H-28	H-28
1′	H_2-10, H-3′,5′	3, H-10, H-10′, H-3′,5′	H-10, H-10′, H-3′,5′	H-10, H-10, H-3′,5′
2′,6′	H_2-10	H-3′,5′, H-4′	H-10, H-10′, H-3′,5′, H-4′	H-10, H-10′, H-3′,5′, H-4′
3′,5′		H-10, H-10′	H-10, H-10′,	
4′	H-2′,6′, H-3′,5′		H-2′,6′	H-2′,6′
Me-16			H-17, H-17′	
OMe				

Northern Osetia (Russia), and Oslo (Norway), and identified as *Ascochyta sonchi* (Sacc.) Grove according to Mel'nik,[8] subsequently renamed *Phoma exigua* Desm. var. *exigua*.[9] Fungal strains were maintained on agar slants (PDA) at 5 °C and deposited in the collection of the All-Russian Institute of Plant Protection (St. Petersburg, Russia) with the internal number C-177. For conidial production, the strains was grown on malt extract agar (Difco, Detroit, USA), or oatmeal agar[31] at 24±2 °C, first for 4 days in the dark and then for 10 days under alternate near-UV light (14 h light/day) and dark. Under these conditions fungal colonies sporulated abundantly. The conidia were rinsed from the agar slants by adding sterile water (containing 0.01% Tween-20). Spore suspensions were then filtered through cheesecloth and the conidial concentrations were adjusted to 1×10^7 conidia/mL. Measurements,

Table 4
2D ^{1}H NOE (NOESY) data obtained for phomachalasins A–D (**1**–**4**)

1		**2**		**3**		**4**	
Considered	Effects	Considered	Effects	Considered	Effects	Considered	Effects
H-3	H-4, H_3-11, H-2′,6′	H-4	H-8, H_3-11	H-3	H-4, H_3-11	H-3	H_3-11
H-4	H_3-11	H-5	H_3-11	H-4	H_3-11	H-5	H_3-11
H_3-11	H-4, H-3	H-7	H-8, H-12, H-12′, H-13	H-5	H_3-11	H-10	H_3-11
H-10	H-2′,6′	H-8	H-4, H_3-11	H-7	H-8	H-10′	H_3-11
H-10′	H-2′,6′	H-10	H-4, H_3-11	H-10	H-10′	H_3-11	H-3, H-5, H-10, H-10′
H-12	H_3-11	H-10′	H-4, H_3-11	H-10′	H-10, H_3-11	H-12	H-7
H-12′	H_3-11	H-12	H-7	H-12′	H_3-11	H-12′	H_3-11
H-13	H-15′	H-12′	H_3-11	H-13	H-7, H-15′	H-13	H-15
H-14	H-8, H-15, Me-16	H-13	H-7, H-8, H-15′	H-14	H-8, H-15, H-17	H-14	H-8
H-15	Me-16	H-14	H-15, H-15′, H-16	H-15	H15′, H-16, Me-16	Me-16	H-15, H-15′
H-19	H_2-18	H-15	H-16, Me-16	H-23	H-28, H-29, H-30	H-27	H-19, H-19′, H-28
H-19′	H-17	H-15′	Me-16	H-27	H-28	H-28	H-19, H-30
H-27	H_2-18, H-19′, OMe, H-28	H-23	H-28, H-29, H-30	H-28	H-17, H-17′, H-18, H-19′, H-29	H-30	H-19, H-28
H-29	H-15, H-23, H-30,	H-24	H-18	H-29	H-18′, H-28, H-30	H-2′,6′	H-3, H-10, H-10′, NH
NH_2	H-19, H-15, H-15′	H-27	H-28	H-30	H-19, H-29		
OMe	H-27	H-28	H-29, H-30	H-2′,6′	H-3, H-10, H-10′, H_3-11, NH		
		H-29	H-30	NH	H-3, H-10		
		H-30	H-28, H-15				
		NH	H-3, H-5, H-4, H-15				

description of fungal colonies, and the NaOH spot test were made using the *Phoma* manual.[9]

4.3. Production, extraction, and purification of phomochalasins A–D (1–4)

Roux bottles (2 L) containing 300 mL of a modified M-1 D medium[32] consisted of $Ca(NO_3)_2$, 1.2 mM; KNO_3, 0.79 mM; KCl, 0.87 mM; $MgSO_4$, 3.0 mM; NaH_2PO_4, 0.14 mM; sucrose, 87.6 mM; ammonium tartrate, 27.1 mM; $FeCl_3$, 7.4 μM; $MnSO_4$, 30 μM; $ZnSO_4$, 8.7 μM; H_3BO_3, 22 μM; and KI, 4.5 μM, (pH was adjusted to 5.5 with 0.1 M HCl) were inoculated with 0.3 mL of a conidial suspension of the strain C-177 (approximately 10^7 conidia/mL). After 4 weeks' incubation under static conditions at 25 °C in the dark, cultures were filtered and then the liquid phase extracted with EtOAc (3×500 mL). The organic extracts were combined, dried (Na_2SO_4), filtered, and evaporated under reduced pressure to give an oily residue (101.0 mg).

Strains C-177 was also grown on autoclaved millet in ten 1000-mL Erlenmeyer flasks (millet 100 g, water 60 mL) for 14 days in the dark. Fungal metabolites were extracted from dry mycelium according to a slightly modified protocol of Evidente et al.[25] The dried material (800 g) was extracted with the acetone–water mixture (1:1, 2 L). After evaporation of acetone, NaCl (300 g/L) was added to the aqueous residue, and the latter was extracted with EtOAc (3×500 mL). The organic extracts were combined, dried (Na_2SO_4), and evaporated under reduced pressure yielding 1.43 g of a brown oily residue, which proved to be highly phytotoxic when assayed on detached thistle leaves as described below. This organic extract was fractionated by column chromatography (eluent A), yielding 10 groups of homogeneous fractions. The residue of the second fraction (103.6 mg) was further fractionated by column chromatography (eluent B) yielding seven groups of homogeneous fractions. The residue of the fourth fraction (33.0 mg) was purified by preparative TLC (eluent C) yielding five homogeneous fractions. The most polar of these (17.4 mg) was further purified by preparative TLC (eluent A) to give a pure metabolite as a crystalline solid named phomachalasin A (1, R_f 0.14, mg 11.6, 14.5 mg/kg). The residues of the third (329.8 mg) and fourth (244.0 mg) fraction groups of the initial column were crystallized separately twice from EtOAc–*n*-hexane (1:5 v/v), giving white needles of cytochalasin B (5, 220 and 200 mg, respectively, 525 mg/kg). The mother liquors (77.5 and 22.7 mg, respectively) of cytochalasin B crystallization were combined and fractionated by column chromatography (eluent B), yielding eight groups of homogeneous fractions. The residue of the third fraction (4.9 mg) was further purified by preparative TLC (eluent B) yielding a pure metabolite as an amorphous solid named phomachalasin C (3, R_f 0.41, 2.6 mg, 3.3 mg/kg). The residue of the fifth fraction (32.5 mg) of the same column chromatography was fractionated by preparative TLC (eluent C) yielding three groups of fractions. The least polar of these fractions (4.6 mg) was further purified by preparative TLC (eluent B) yielding cytochalasin F as an amorphous solid.[15] The most polar fraction (9.6 mg) of the last TLC was finally purified by preparative TLC (eluent A), yielding a further amount of phomachalasin A (1) as an amorphous solid (4.5 mg, for a total of 20.1 mg, 25.1 mg/kg). The sixth fraction (8.6 mg) of the last column chromatography was purified by preparative TLC (eluent C) yielding two pure compounds as an amorphous solid: deoxaphomin[15] and a pure metabolite as an amorphous solid named phomachalasin B (2, R_f 0.16, 2.5 mg, 3.1 mg/kg).

The organic extracts (100 mg) obtained from *P. exigua* var. *exigua* liquid culture (1 L of M1-D) was fractionated by column chromatography (eluent A), yielding nine groups of homogeneous fractions. The residue of the second fraction (13.6 mg) was further purified by preparative TLC (eluent B) yielding three pure compounds as an amorphous solid: *p*-hydroxybenzaldehyde (R_f 0.62, 1.0 mg), cytochalasin B (R_f 0.25, 2.2 mg), and another metabolite identified as phomachalasin B (**2**, R_f 0.18, 1.2 mg). The third fraction (12.3 mg) of the first column was purified by preparative TLC (eluent A) yielding six homogeneous fractions. The most abundant of them (5.2 mg) was finally purified by preparative TLC on reversed phase (eluent D) yielding a pure compound as an amorphous solid named phomachalasin D (**4**, R_f 0.52, 0.8 mg).

*4.3.1. Phomachalasin A (**1**).* Compound **1**: $[\alpha]_D^{25}$ −13 (*c* 0.1); ν_{max} 3383, 1705,1615, 1516, 1455 cm^{-1}; λ_{max} (ε) 282 (891), 276 (1055), 226 (sh); ^1H and ^{13}C NMR spectra: see Tables 1 and 2; *m*/*z* (ESI) 1371 ($2MNa^+$); HRMS (ESI): MNa^+, found 697.3099, $C_{38}H_{46}N_2NaO_9$ requires 697.3101.

*4.3.2. Phomachalasin B (**2**).* Compound **2**: $[\alpha]_D^{25}$ −73 (*c* 0.1); ν_{max} 3379, 1704, 1696, 1613, 1572, 1514, 1456 cm^{-1}; λ_{max} (ε) 282 (823), 275 (932), 225 (sh); ^1H and ^{13}C NMR spectra: see Tables 1 and 2; *m*/*z* (ESI) 697 (MK^+); HRMS (ESI): MNa^+, found 681.3150, $C_{38}H_{46}N_2NaO_8$ requires 681.3153.

*4.3.3. Phomachalasin C (**3**).* Compound **3**: $[\alpha]_D^{25}$ +33 (*c* 0.2); ν_{max} 3352, 1708, 1618, 1517, 1456 cm^{-1}; λ_{max} (ε) 282 (1118), 276 (1325) 226 (sh); ^1H and ^{13}C NMR spectra: see Tables 1 and 2; *m*/*z* HRMS (ESI): MNa^+, found 697.3103, $C_{38}H_{46}N_2NaO_9$ requires 697.3101.

*4.3.4. Phomachalasin D (**4**).* Compound **4**: ν_{max} 3348, 1710, 1620, 1522, 1458 cm^{-1}; λ_{max} (ε) 282 (118), 276 (1325) 226 (sh); ^1H and ^{13}C NMR spectra: see Tables 1 and 2; *m*/*z* HRMS (ESI): MNa^+, found 697.3098, $C_{38}H_{46}N_2NaO_9$ requires 697.3101.

4.4. Phytotoxic activity

Culture filtrates, organic extracts, their chromatographic fractions, and pure phomachalasins A–D (**1**–**4**) were assayed on leaves of *C. arvense*, *Lycopersicon esculentum* and *Elytrigia repens* by puncture assay. The pure toxins and the fractions were first dissolved in a small amount of ethanol and then diluted to the desired concentration with distilled water (the final ethanol concentration was 5%). Droplets (10 μL) of the assay solutions were applied to punctured detached leaf segments or disks, that were then kept in moistened chambers under continuous light. Symptom appearance was observed 3 days after droplet application. Phomachalasins **1**–**4** were tested at concentrations of around 3×10^{-3} M (2 mg/mL).

4.5. Antimicrobial activity

The antifungal activity of compounds **1**–**4** was tested up to 100 μg/disk on *Candida tropicalis*, whereas the antibiotic activity was assayed on *Bacillus subtilis* as previously described.[3] Briefly, methanolic solutions of the testing compounds were adsorbed on 6 mm concentration disks. After solvent evaporation, disks were laid on potato-dextrose agar (PDA) and sprayed with a mycelium suspension of *C. tropicalis*. Antifungal activity was evaluated after 24 h by the fungal growth inhibition halo. Antibiotic activity was tested using the same method but PDA plates containing the disks adsorbed with the substances were inoculated with a suspension of *B. subtilis*.

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References and notes

1. Evidente, A.; Cimmino, A.; Berestetskiy, A.; Mitina, G.; Andolfi, A.; Motta, A. *J. Nat. Prod.* **2008**, *71*, 31–34.
2. Evidente, A.; Cimmino, A.; Berestetskiy, A.; Andolfi, A.; Motta, A. *J. Nat. Prod.* **2008**, *71*, 1897–1901.
3. Evidente, A.; Andolfi, A.; Abouzeid, M.; Vurro, M.; Zonno, M. C.; Motta, A. *Phytochemistry* **2004**, *65*, 475–480.
4. Evidente, A.; Cimmino, A.; Andolfi, A.; Vurro, M.; Zonno, M. C.; Cantrell, C. L.; Motta, A. *Tetrahedron* **2008**, *64*, 1612–1619.
5. Evidente, A.; Cimmino, A.; Andolfi, A.; Vurro, M.; Zonno, M. C.; Motta, A. *J. Agric. Food Chem.* **2008**, *56*, 884–888.
6. Evidente, A.; Andolfi, A.; Cimmino, A. *Pest Technol. Rev.* **2011**, *5* (Special issue 1).
7. Evidente, A.; Punzo, B.; Andolfi, A.; Berestetskiy, A.; Motta, A. *J. Agric. Food Chem.* **2009**, *57*, 6656–6660.
8. Mel'nik, V. A. Key to the fungi of the genus *Ascochyta* Lib. (Coelomycetes). Hrsg. von der Biologischen Bundesanstalt für Land- und Forstwirtschaft In *Berlin und Brauscheig (Mittteilungen aus der Biol. Bund. für Land- und Forstwirtschaft Berlin-Dahlem)*; Mel'nik, V. A., Braun, U., Hagedorn, G., Eds.; Parey: Berlin, 2000; H. 379.
9. Boerema, G. H.; de Gruyter, J.; Noordeloos, M. E.; Hamers, M. E. C. *Phoma Identification Manual: Differentiation of Specific and Infraspecific Taxa in Culture*; CABI publishing: Wallingford, Oxon, UK, 2004.
10. van der aA, H. A.; Boerema, G. A.; de Gruyter, J. *Personia* **2000**, *17*, 435–456.
11. Holm, L.; Plucknett, D. L.; Pancho, J.; Herberger, J. *The World Worst Weeds*; University: Hawaii, US, 1977.
12. Holm, L.; Doll, J.; Holm, E.; Pancho, J.; Herberger, J. *World Weeds: Natural Histories and Distribution*; John Wiley: New York, NY, 1997, pp 1–129.
13. Berestetskiy, A.O. In *Efficacy of Strains of Different Fungal Species and their Application Techniques for Biological Control of Cirsium arvense*, Proceedings of 2nd Conference on Plant Protection; Pushkin, Saint Petersburg, Russia, December 5–10, 2005; pp 136–138.
14. Berestetskiy, A.O.; Gagkaeva, T.Y.; Gannibal, P.B.; Gasich, E.L.; Kungurtseva, O.V.; Mitina, G.V.; Yuzikhin, O.S.; Bilder, I.V.; Levitin, M.M. In *Evaluation of Fungal Pathogens for Biocontrol of Cirsium arvense*, Proceedings of 13th European Weed Research Society Symposium; Bari, Italy, June 19–23, 2005; Abstract 7.
15. Cimmino, A.; Andolfi, A.; Berestetskiy, A.; Evidente, A. *J. Agric. Food Chem.* **2008**, *56*, 6304–6309.
16. Capasso, R.; Evidente, A.; Randazzo, G.; Ritieni, A.; Bottalico, A.; Vurro, M.; Logrieco, A. *J. Nat. Prod.* **1987**, *50*, 989–990.
17. Capasso, R.; Evidente, A.; Vurro, M. *Phytochemistry* **1991**, *30*, 3945–3950.
18. Capasso, R.; Evidente, A.; Ritieni, A.; Randazzo, G.; Vurro, M.; Bottalico, A. *J. Nat. Prod.* **1988**, *51*, 567–571.
19. Nakanishi, K.; Solomon, P. H. *Infrared Absorption Spectroscopy*, 2nd ed.; Holden Day: Oakland, 1977, pp 17–44.
20. Scott, A. *Interpretation of the Ultraviolet Spectra of Natural Products*; Pergamon: Oxford, 1964, pp 45–88.
21. Pretsch, E.; Bühlmann, P.; Affolter, C. *Structure Determination of Organic Compounds—Tables of Spectral Data*; Springer: Berlin, 2000, pp 161–243.
22. Berger, S.; Braun, S. *200 and More Basic NMR Experiments: a Practical Course*, 1st ed.; Wiley-VCH: Weinheim, 2004.
23. Breitmaier, E.; Voelter, W. *Carbon-13 NMR Spectroscopy*; VCH: Weinheim, 1987, pp 183–280.
24. Vurro, M.; Bottalico, A.; Capasso, R. In *Evidente in Toxins in Plants Disease Development and Evolving Biotechnology*; Upadahyay, R. K., Mukerji, K. G., Eds.; Oxford & IBH publishing: New Delhi, 1997; pp 127–147.
25. Evidente, A.; Andolfi, A.; Vurro, M.; Zonno, M. C.; Motta, A. *Phytochemistry* **2002**, *60*, 45–53.
26. Evidente, A.; Andolfi, A.; Vurro, M.; Zonno, M. C.; Motta, A. *J. Nat. Prod.* **2003**, *66*, 1540–1544.
27. Bottalico, A.; Capasso, R.; Evidente, A.; Randazzo, G.; Vurro, M. *Phytochemistry* **1990**, *29*, 93–96.
28. Berestetskiy, A.; Dimitriev, A.; Mitina, G.; Lisker, I.; Andolfi, A.; Evidente, A. *Phytochemistry* **2008**, *69*, 953–960.
29. Carter, S. B. *Nature* **1967**, *213*, 261–264.
30. Cole, J. C.; Cox, R. H. *Handbook of Toxic Fungal Metabolite*; Academic: New York, NY, 1981; 264–343.
31. Ritchie, B. J. In *Mycological Methods and Media. Plant Pathologist's Pocketbook*; Waller, J. M., Lenné, J., Waller, S. J., Eds.; CABI International: Egham, UK, 2002.
32. Pinkerton, F.; Strobel, G. A. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 4007–4011.