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Phomentrioloxin: A Phytotoxic Pentasubstituted Geranylcyclohexentriol Produced by *Phomopsis* sp., a Potential Mycoherbicide for *Carthamus lanatus* Biocontrol

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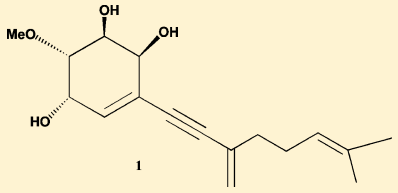
Supporting Information

ABSTRACT: A new phytotoxic geranylcyclohexenetriol, named phomentrioloxin, was isolated from the liquid culture of *Phomopsis* sp., a fungal pathogen proposed for the biological control of *Carthamus lanatus*, a widespread and troublesome thistle weed belonging to the Asteraceae family causing severe crop and pastures losses in Australia. The structure of phomentrioloxin was established by spectroscopic, X-ray, and chemical methods as (1*S*,2*S*,3*S*,4*S*)-3-methoxy-6-(7-methyl-3-methylene-oct-6-en-1-ynyl)-cyclohex-5-ene-1,2,4-triol. At a concentration of 6.85 mM, the toxin causes the appearance of necrotic spots when applied to leaves of both host and nonhost plants. It also causes growth and chlorophyll content reduction of fronds of *Lemna minor* and inhibition of tomato rootlet elongation. Finally, in preliminary bioassays, phomentrioloxin did not show any antibacterial, fungicidal, or zootoxic activities.



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Saffron thistle (*Carthamus lanatus* L. ssp. *lanatus*) is a widespread winter-growing annual weed of both pastures and crops throughout Australia, introduced from the Mediterranean region.[1] It is considered the most economically important thistle species in New South Wales[2,3] and was one of the weeds targeted by the Australian Cooperative Research Centre for Weed Management Systems.[4] It is declared noxious in all Australian States.[5]

Poor results of mechanical[5] and chemical control have made this weed a suitable target for biological control.[2] In an effort to develop a mycoherbicide against this weed, a number of pathogenic isolates of *Phomopsis* have been identified from naturally infected saffron thistle plants in Australia,[6] and their potential as mycoherbicides against the host evaluated.[7] Furthermore, a recent study on genetic diversity carried out on a large number of strains supported the hypothesis that the *Phomopsis* sp. pathogenic to saffron thistle can be considered a new species.[8] Considering that the fungus causes elliptical necrosis on the stems resulting in plant death, the involvement of phytotoxins produced by the fungus in the disease development process has been assumed. Previous studies proved the inability of these strains[9] to produce the powerful mammalian mycotoxin phomopsin A, which is indeed produced by other *Phomopsis* sp.,[10] but did not aim to ascertain the eventual production of other bioactive metabolites.

Considering that phytopathogenic fungi are important sources of bioactive metabolites having herbicidal potential,[11,12] and considering that species of the genus *Phomopsis* are well known to be producers of phytotoxic metabolites,[13,14] studies were carried out in order to ascertain the capability of this *Phomopsis* sp. to produce novel bioactive metabolites.

This article reports on (a) the isolation and the chemical characterization of the main phytotoxin produced by one strain of *Phomopsis* sp. isolated from *Carthamus lanatus*; (b) the preliminary studies of its biological properties in order to evaluate its potential to be developed as a natural and safe herbicide.

RESULTS AND DISCUSSION

The organic extract of *Phomopsis* sp., showing a high phytotoxic activity, was purified by a combination of CC and TLC guided by biological assays as reported in detail in the Experimental Section. The main phytotoxic metabolite (10 mg/L) was obtained as a homogeneous solid, which crystallized as white needles from MeOH. The preliminary ^{1}H and ^{13}C NMR investigations proved that it contains double bonds and

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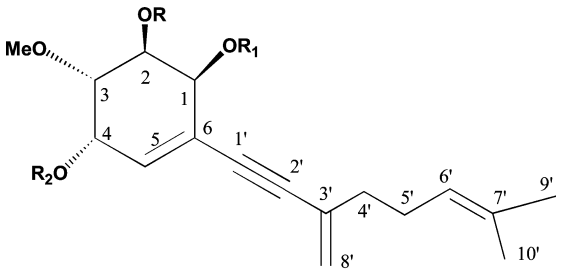
hydroxy groups and that it is a novel metabolite, to which the name phomentrioloxin was assigned (**1**, Figure 1).



1 R=R_1=R_2=H
2 R=R_1=R_2=Ac
3 R+R_1=Me_2C, R_2=H
4 R+R_1=Me_2C, R_2=*S*-MPTA
5 R+R_1=Me_2C, R_2=*R*-MPTA

Figure 1. Structures of phomentrioloxin (**1**), its 1,2,4-*O*,*O*′,*O*″-triacetyl (**2**) and 1,2-*O*,*O*′-isopropylidene (**3**) derivatives, and the 4-*O*-*S*- and -*R*-MPTA esters of the 1,2-*O*,*O*′-isopropylidene derivative of phomentrioloxin (**4** and **5**, respectively).

Phomentrioloxin has a molecular weight 292, due to its molecular formula $C_{17}H_{24}O_4$ deduced by its HRESIMS, consistent with six hydrogen deficiencies. The latter were also confirmed by the typical bands observed in the IR spectrum for the olefinic groups and hydroxy groups. The same spectrum also showed a typical band for the presence of at least one alkyne group.[15] The UV spectrum showed absorptions maxima of an extended conjugated chromophore.[16]

The detailed investigation of the ^{1}H NMR spectrum (Table 1) showed the presence of an olefinic proton (H-5) appearing (d, J = 4.0 Hz) at the typical chemical value of δ 6.16.[17] In the COSY spectrum[18] the latter coupled with a proton (H-4) of the secondary hydroxylated carbon (C-4) resonating as doublets of doublet doublets (J = 6.1, 4.1, and 4.0 Hz) at δ 4.51, being also coupled with the protons of the geminal hydroxy group (a doublet, J = 6.1 Hz) at δ 2.64 and the proton (H-3) of another adjacent secondary oxygenated carbon (C-3). H-3, resonating as a double doublet (J = 7.9 and 4.1 Hz) at δ 3.70, in turn coupled with the proton (H-2) of another adjacent hydroxylated secondary carbon (C-2) appearing as doublets of double doublets (J = 7.9, 3.6, and 2.0 Hz) at δ 4.21. The latter also coupled with the proton of the geminal hydroxy group (d, J = 2 Hz) at δ 2.68 and with the proton (H-1) of the third adjacent secondary hydroxylated carbon (C-1). H-1 appeared as a broad singlet at δ 4.35. The same ^{1}H NMR spectrum also showed a further doublet (J = 1.4 Hz) of a hydroxy group and the singlet of a methoxy group linked to C-1 and C-3 at δ 2.65 and 3.55, respectively.

Table 1. ^{1}H and ^{13}C NMR Data of Phomentrioloxin (1)[a,b]

position	δ_C[c]	δ_H (J in Hz)	HMBC
1	69.1 CH	4.35 (1H) br s	H-5, H-3, HO-1
2	67.9 CH	4.21 (1H) ddd (7.9, 3.6, 2.0)	
3	79.1 CH	3.70 (1H) dd (7.9, 4.1)	H-5, OMe, OH-2
4	64.8 CH	4.51 (1H) ddd (6.1, 4.1, 4.0)	
5	135.3 CH	6.16 (1H) d (4.0)	
6	135.4 C		H-1
1′	87.3 C		H-5, H-1
2′	92.4 C		H_2-8′
3′	131.5 C		H_2-8′, H_2-4′
4′	37.9 CH_2	2.22 (2H) m	
5′	18.4 CH_2	1.64 (2H) m	H_2-8′
6′	124.4 CH	5.13 m	Me-10′, Me-9′
7′	123.1 C		H_2-4′, H_2-5′
8′	123.8 CH_2	5.40 (1H) d (1.5)	
		5.31 (1H) d (1.5)	
9′[d]	27.4 CH_3	2.22 (3H) s	H-6′, Me-10′
10′[d]	26.4 CH_3	1.85 (3H) s	Me-9′
OMe	59.3 CH_3	3.55 (3H) s	
HO-1		2.65 (1H) d (1.4)	
HO-2		2.68 (1H) d (2.0)	
HO-4		2.64 (1H) d (6.1)	

[a]Chemical shifts are in δ values (ppm) from TMS. [b]2D ^{1}H,^{1}H (COSY) and ^{13}C,^{1}H (HSQC) NMR experiments delineated the correlations of all the protons and the corresponding carbons. [c]Multiplicities were assigned by DEPT spectrum. [d]These assignments may be reversed.

The structure assigned to phomentrioloxin was further confirmed by preparing two key derivatives, which were also used in the bioassay described below, comparing their phytotoxicity to that of **1**. By acetylation and acid-catalized ketalization, **1** was converted into 1,2,4-*O*,*O*′,*O*″-triacetyl- (**2**) and 1,2-*O*,*O*′-isopropylidene (**3**) derivatives, respectively.

These results agreed with the presence in **1** of a 1,2,4-trihydroxy-3-methoxycyclohexene ring. Consequently, the polyunsaturated side chain constituted by the remaining 10 carbons should be attached to C-6 of this pentasubstituted cyclohexene ring. This suggested a terpenoidal origin of this second partial moiety. Indeed, the ^{1}H NMR and COSY spectra showed the presence of (1) two coupled doublets (J = 1.5 Hz) at δ 5.40 and 5.31, respectively, representing typical chemical shift values for protons of an olefinic methylene group (H_2C-8′); (2) the multiplets of two coupled methylene groups (H_2C-4′ and H_2C-5′) at δ 2.22 and 1.64, respectively; and (3) singlets of two vinylic methyl groups (Me-9′ and Me-10′) at δ 2.22 and 1.85, respectively. The remaining two carbons of the geranyl residue attached to C-6 of the cyclohexene ring as well as the latter carbon are quaternary carbons resonating in the ^{13}C NMR spectrum (Table 1) at δ 135.4, 87.3, and 92.4 (C-6, C-1′, and C-2′), typical chemical shift values of olefinic and alkyne carbons.[19] The geranyl nature of the side chain attached was delineated by several long-range couplings observed between the carbons and the protons of this moiety, as observed in the HMBC spectrum (Table 1). The presence of these two joined moieties in **1** was confirmed by the other data of its ^{13}C NMR spectrum (Table 1). Indeed the latter spectrum showed the presence of five methine carbons at δ 135.3, 79.1, 69.1, 67.9, and 64.8 (C-5, C-3, C-1, C-2, and C-4, respectively); three methylenes at δ 123.8, 37.9, and 18.4 (C-8′, C-4′, and C-5′, respectively); two methyls at δ 27.4 and 26.4 (C-9′ and C-10′, respectively); and one methoxy carbon at δ 59.3 (OMe). They were assigned on the basis of the couplings observed in the HSQC spectrum.

On the basis of these results the structure of 1,2,4-triol-3-methoxy-6-(7-methyl-3-methyleneoct-6-en-1-ynyl)cyclohex-5-ene was assigned to the metabolite, named phomentrioloxin (**1**).

This structure was supported by several long-range couplings observed in the HMBC spectrum (Table 1) and by the HRESIMS data. Indeed, the latter spectrum showed, besides the dimeric sodium cluster at m/z 607, the potassium and the sodium clusters at m/z 331 and 315.1561, respectively.

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As expected, the IR spectrum of **2** lacked bands due to hydroxy groups. Its ^{1}H NMR spectrum differed from that of **1** essentially by lacking hydroxy group signals, the downfield shifts ($\Delta\delta$ 1.46, 1.27, and 1.22), and the multiplicity of H-1, H-2, and H-4 resonating as a doublet (J = 4.1), a double doublet (J = 8.9 and 4.1 Hz), and a double doublet (J = 4.4 and 4.1 Hz), at δ 5.81, 5.48, and 5.73, respectively. Finally, the presence of the three singlets at δ 2.14, 2.13, and 2.09, respectively, due to the three acetyl groups was also observed. Its ESIMS spectrum showed the sodium cluster at m/z 441.

The ^{1}H NMR data of derivative **3** differed from those of **1**, i.e., lacking the two broad singlets of the two hydroxy group at C-1 and C-2 and in the presence of two singlets of the isopropylidene group at δ 1.43 and 1.40, respectively. Furthermore, the spectrum indicated downfield shifts ($\Delta\delta$ 0.22 and 0.27) of the doublet (J = 5.7 Hz) and a double doublet (J = 5.7 and 4.6 Hz) of H-1 and H-2 resonating at δ 4.57 and 4.48 as engaged in the isopropylidene ring. The ESIMS data showed the sodium cluster of both **3** and its dimer at m/z 355 $[M + Na]^+$, 687 $[2M + Na]^+$.

The relative configuration of **1** was deduced from the values measured for coupling between the protons of the cyclohexene ring. On the basis of these values (Table 1), H-2 and H-3, and H-1 and H-4, were pseudoaxially and pseudoequatorially located, while the cyclohexene ring probably assumes a twisted conformation, as also observed by an inspection of a Dreiding model of **1**.[17] This relative configuration as well as the structure assigned to **1** were confirmed by the X-ray analysis (Figure 2).

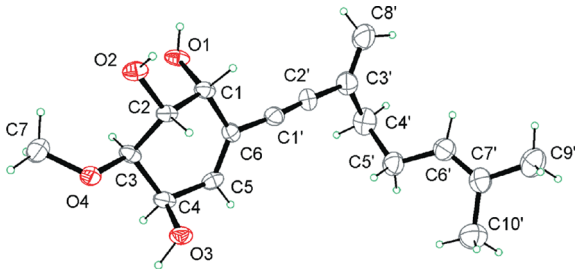

Figure 2. ORTEP view of phomentrioloxin (**1**) showing atomic labeling. Displacement ellipsoids are drawn at the 30% probability level.

Compound **1** crystallizes in the orthorhombic $P2_12_12_1$ space group. An ORTEP view of the molecule is shown in Figure 2. Bond lengths and angles in **1** are in the normal range.[20] In the six-membered ring, the C-5−C-6 bond distance and the geometry around C-5 and C-6 confirmed the presence of the cyclohexene double bond. The cyclohexene ring adopts a twisted conformation with C-2 and C-3 pointing up and down from the plane formed by C-1/C-6/C-5/C-4. The ring geometry is constrained by the presence of the C-5−C-6 double bond. A further structural constraint is represented by a triple bond (C-1′−C-2′) vicinal to the ring and a double bond between C-3′ and C-8′. The two hydroxy groups at C-1 and C-4 are axial and point in opposite directions with respect to the plane of the cyclohexene ring. The third hydroxy group at C-2 is disposed in the equatorial position. The sequence of torsion angles around the C3′−C4′, C4′−C5′, and C5′−C6′ bonds [−67.8(6)°, −71.3(6)°, −172.6(5)°, respectively] of the 7-methyl-3-methyleneoct-6-en-1-ynyl group generates the characteristic V-shape of the molecule. Four stereogenic centers are present in the molecule at C1/C2/C3/C4, whose relative configuration is *R**/*R**/*R**/*R**. It was not possible to determine the absolute configuration, because of weak anomalous scattering. In the crystal packing all OH groups are involved in a pattern of intermolecular OH···O hydrogen bonds.

The relative configuration assigned to **1** was also confirmed by the couplings observed in the NOESY spectrum.[18] The correlation between H-1 and H-2 and that between H-4 and both H-5 and H-3 as well as the lack of correlation between H-2 and H-3 appeared to be particularly significant. Finally, the correlation between the methoxy group and both Me-9′ and Me-10′, in agreement with the inspection of the Dreiding models of **1**, indicated a bending of the side chain toward the cyclohexene ring.

The absolute configuration of **1** was determined by applying an advanced Mosher's method.[21] By reaction with *R*-(−)-α-methoxy-α-trifluoromethylphenylacetate (MTPA) and *S*-(+)MTPA chlorides, the 1,2-*O*,*O*′-isopropylidene derivative (**3**) was converted into the corresponding diastereomeric *S*-MTPA and *R*-MTPA monoesters at C-4 (**4** and **5**, respectively), whose spectroscopic data were consistent with the structure assigned to **1**. Comparison between ^{1}H NMR data (Table 2) of the *S*-MTPA (**4**) and *R*-MTPA (**5**) esters of **1** permitted assignment of the $\Delta\delta$ (**4**, **5**) values of all the protons as reported in Figure 3 and the assignment of the *S*-configuration to C-4. Consequently a 1*S*, 2*S*, and 3*S* configuration was assigned to C-1, C-2, and C-3, respectively, and **1** was formulated as (1*S*,2*S*,3*S*,4*S*)-3-methoxy-6-(7-methyl-3-methyleneoct-6-en-1-ynyl)cyclohex-5-ene-1,2,4-triol.

The closest phytotoxin to phomentrioloxin appears to be foeniculoxin (Table 3), a geranyl hydroquinone isolated as the main bioactive lipophilic metabolite from the culture filtrates of a strain of *Phomopsis phoeniculi*, the causal agent of wilting of stems and inflorescences of fennel.[22] Foeniculoxin belongs to the polyprenylated 1,4-benzoquinone and 1,4-hydroquinone groups of natural compounds, which, like ubiquinone, plastoquinone, and tocopherols, are widespread in plants, animals, and marine organisms, in which they play important roles in electron transport, in photosynthesis, and as antioxidants.[23] Some examples of geranyl-quinones and -hydroquinones isolated from different organisms and with interesting biological activities, structurally related to phomentrioloxin, are reported in Table 3.[24−32] Very significant for the pathological and taxonomical aspects appeared to be the close relation between the structures of the toxins produced by the strain of *P. foeniculi* isolated in Italy and of that produced by the strain of *Phomopsis* sp. isolated in Australia from saffron thistle. A number of *Phomopsis* species have been isolated from fennel in Europe.[33,34] This commonality of toxins between the Australian and European isolates could reflect common origins for these isolates in Europe or could indicate gene flow between species in the region.

A broad number of different biological assays were performed with the main toxin and two derivatives. The leaf puncture assay was carried out also with the extracts and the fractions for guiding the purification procedures. These assays, summarized in Table 4, aimed at obtaining preliminary information on (1) the phytotoxicity of the compound; (2) the potential for its use as a natural herbicide; (3) the impact on nontarget organisms; and (4) preliminary observations on structure−activity relationships.

Assayed on leaves of several weeds at 40 μg/droplet (6.85 mM), phomentrioloxin caused the appearance of necrotic

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Table 2. ^{1}H NMR Data ($CDCl_3$) of 1,2,4-*O*,*O*′,*O*″-Triacetyl- and 1,2-*O*,*O*′-Isopropylidene and 4-*O*-*S*- and 4-*O*-*R*-MTPA Esters of Phomentrioloxin (2, 3, 4 and 5, respectively)[a]

	2	3	4	5
position	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)
1	5.81 (1H) d (4.1)	4.57 (1H) d (5.7)	4.609 (1H) d (6.1)	4.575 (1H) d (6.1)
2	5.48 (1H) dd (8.9, 4.1)	4.48 (1H) dd (5.7, 4.6)	4.249 (1H) dd (7.1, 6.1)	4.317 (1H) dd (7.1, 6.1)
3	3.81 (1H) dd (8.9, 4.1)	3.67 (1H) t (4.6)	3.592 (1H) dd (7.1, 3.5)	3.612 (1H) dd (7.1, 3.3)
4	5.73 (1H) dd (4.4, 4.1)	4.39 (1H) ddd (8.4, 4.6, 3.3)	5.835 (1H) dd (5.0, 3.5)	5.799 (1H) dd (5.2, 3.3)
5	6.15 (1H) d (4.4)	6.11 (1H) d (3.3)	6.151 (1H) d (5.0)	6.136 (1H) d (5.2)
1′				
2′				
3′				
4′	1.45 (2H) m	2.20 (2H) m	2.210 (2H) m	2.203 (2H) m
5′	1.32 (2H) m	1.56 (2H) m	1.547 (2H) m	1.551 (2H) m
6′	5.09 (1H) m	5.11 (1H) m	5.108 (1H) m	5.105 (1H) m
7′				
8′	5.35 (1H) d (1.7)	5.36 (1H) d (1.5)	5.406 (1H) d (1.6)	5.399 (1H) d (1.0)
	5.30 (1H) d (1.7)	5.26 (1H) d (1.5)	5.309 (1H) d (1.6)	5.305 (1H) d (1.0)
9′[b]	1.70 (3H) s	1.69 (3H) s	1.688 (3H) s	1.686 (3H) s
10′[b]	1.62 (3H) s	1.62 (3H) s	1.620 (3H) s	1.616 (3H) s
OMe	3.49 (3H) s	3.54 (3H) s	3.549 (3H) s	3.592 (3H) s
HO-4		2.51 d (1H) (8.4)		
MeCO	2.14 (3H) s, 2.13 (3H) s, 2.09 (3H) s			
Me_2C		1.43 (3H) s and 1.40 (3H) s	1.482 (3H) s and 1.380 (3H) s	1.498 (3H) s and 1.383 (3H) s
OMe			3.386 (3H) s	3.491 (3H) s
Ph			7.700−7.506 (5H) m	7.705−7.401 (5H) m

[a]Chemical shifts are in δ values (ppm) from TMS. [b]These assignments may be reversed.

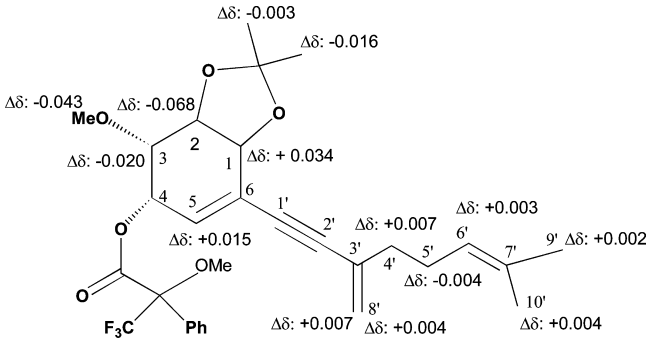

Figure 3. Structure of the 4-*O*-*S*- and -*R*-MPTA esters of the 1,2-*O*,*O*′-isopropylidene derivative of phomentrioloxin (**4** and **5**, respectively) reporting the $\Delta\delta$ value obtained by comparison (**4** − **5**) of each proton system.

lesions one day after the application. A large area of necrosis (around 1 cm diameter) was particularly evident on the leaves of *Cirsium arvense* and *Sonchus oleraceus*. Slightly smaller necrotic areas (6−8 mm) were clearly observable on leaves of the host plant *C. lanatus*, but also on leaves of the other two dicotyledonous weed species tested, i.e., *Mercurialis annua* and *Chenopodium album*. Necrotic spots were smaller (diameter around 3 mm) on *Setaria viridis* (monocot). Assayed at 3.42 mM, phomentrioloxin caused clearly observable lesions on *C. arvense*, *S. oleraceus*, and the host as well, whereas at 1.71 mM, it was active only on *C. arvense* leaves. At the highest concentration tested the 1,2-*O*,*O*′-isopropylidene derivative **3** proved to be slightly active against the dicotyledonous weeds and inactive against *S. viridis*, whereas 1,2,4-*O*,*O*′,*O*″-triacetyl derivative **2** was completely inactive against all plants tested.

Assayed on fronds of *Lemna minor* at 6.85 mM the main toxin caused approximately 90% reduction of the chlorophyll content and 50% reduction of the fresh weight of the fronds (Table 4). At a concentration 10 times lower the metabolite had a modest and not significant toxicity, whereas at concentrations even lower it was completely inactive (data not shown).

Tested at 1.37 mM on germinated tomato seeds, phomentrioloxin caused around 50% inhibition of rootlet elongation (Table 4), whereas the two derivatives had almost no activity (data not shown).

These results suggested that all the hydroxy groups of the cyclohexene ring are important features for the phytotoxicity. However, the role of the geranyl side chain and its functionalities remain to be investigated.

When phomentrioloxin was assayed at 3.42 mM, only around 32% protoplasts of *Arabidopsis thaliana* remained viable (Figure 4), compared to 67% cell viability of the control cells. At 0.34 mM the toxin was still active, whereas at lower concentrations its toxicity was progressively reduced (Figure 4).

Interestingly, phomentrioloxin and the two derivatives proved to be inactive in the other preliminary bioassays carried out on organisms other than plants. In particular, they proved to be inactive when tested for antifungal activity against *Geotrichum candidum* and for antibacterial activity against *Bacillus subtilis* and *Escherichia coli* up to 100 μg/diskette. Moreover the main toxin and the two derivatives caused no larvae mortality when supplied to shrimps through artificial seawater at concentrations up to 0.171 mM.

The wide range of preliminary bioassays performed seems to show that phomentrioloxin, the main metabolite produced by the fungus, has only phytotoxic properties and no toxicity to other nontarget organisms at the concentrations tested. This characteristic, to be further confirmed, could be particularly important in the attempts of finding novel metabolites to be used as natural and safe herbicides. The phytotoxicity is not

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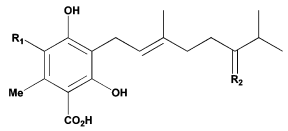
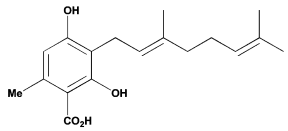
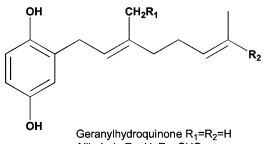
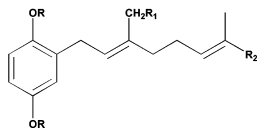
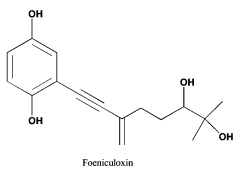
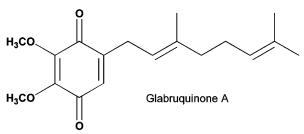
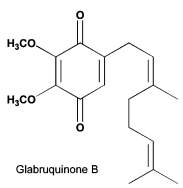
Table 3. Natural Compounds Structurally Related to Phomentrioloxin

Structure/Name	Compound source	Biological Activity	Literature
 Colletochlorin, R_1=Cl, R_2= H,OH Colletochlorin C, R_1=Cl, R_2=O Colletorin A, R_1=H, R_2=H,OH Colletorin C , R_1=H, R_2=O	*Colletotrichum nicotianae*	Inductor of differentiation of human promyelocytic leukemia cells. Inhibition of acetylcholinesterase and β-glucuronidase; inhibition of seed germination, and radicle and epicotyl growth	Kosuge *et al.*, 1973, [24] Kosuge *et al.*, 1974[25] Gutierrez et al., 2005[26]
 Geranylorsellinic acid	*Helminthosporium siccans*	Not reported	Suzuki and Nozoe 1978[27] Takahashi *et al.*, 1988[28]
 Geranylhydroquinone R_1=R_2=H Alliodorin R_1=H, R_2=CHO Alliodorol, R_1=H,, R_2=CH_2OH Cordalinol, R_1=OH R_2=CH_2OH	*Cordia alliodora*	Low activity against marine organisms and wood destroying fungi	Manners *et al.*, 1977[29]
 Methyl alliodorate, R=R_1=H, R_2=CO_2Me Cordallinal R=H, R_1=OH, R_2=CHO	*Cordia elaeagnoides*	Not reported	Manners, 1983[30]
 Foeniculoxin	*Phomopsis foeniculi*	Phytotoxicity	Evidente *et al.*,[22]
 Glabruquinone A  Glabruquinone B	*Aplidium glabrum*	Cancer preventives properties	Shubina *et al.*, 2005;[31] Federov *et al.*, 2006[32]

particularly strong, considering that clear necrosis or effects on cells and chlorophyll could be observed only when using relatively high concentrations of toxin. On the hand, the lack of antifungal and antibacterial activities seems to be quite evident, as the amount of toxin used in those assays was biologically high. Moreover, the preliminary studies on structure−activity relationships showed that structural modifications could modify biological activities and phytotoxicity. These aspects will be addressed in the future for a better assessment of the potential

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Table 4. Biological Activities of Phomentrioloxin (see Experimental Section for details)

organism	bioassay/application	toxin concentration	type of effect measured	activity
Carthamus lanatus	leaf puncture	6.85×10^{-3} M	necrosis	6–8 mm
Chenopodium album	leaf puncture	6.85×10^{-3} M	necrosis	6–8 mm
Cirsium arvense	leaf puncture	6.85×10^{-3} M	necrosis	1 cm
Mercurialis annua	leaf puncture	6.85×10^{-3} M	necrosis	6–8 mm
Sonchus oleraceus	leaf puncture	6.85×10^{-3} M	necrosis	1 cm
Setaria viridis	leaf puncture	6.85×10^{-3} M	necrosis	2–3 mm
Arabidopsis thaliana	protoplasts	3.42×10^{-3} M	cell death	51% (±3)
Lemna minor	frond immersion	6.85×10^{-3} M	chlorophyll reduction	94% (±3)
Lemna minor	frond immersion	6.85×10^{-3} M	rootlet growth inhibition	47% (±2)
Lycopersicon esculentum	germinated seeds	1.37×10^{-3} M	rootlet growth inhibition	50% (±9)
Escherichia coli	agar diffusion	up to 100 μg/disk	inhibition halo	inactive
Bacillus subtilis	agar diffusion	up to 100 μg/disk	inhibition halo	inactive
Geotrichum candidum	agar diffusion	up to 100 μg/disk	inhibition halo	inactive
Artemia salina	larvae	1.71×10^{-4} M	mortality	inactive

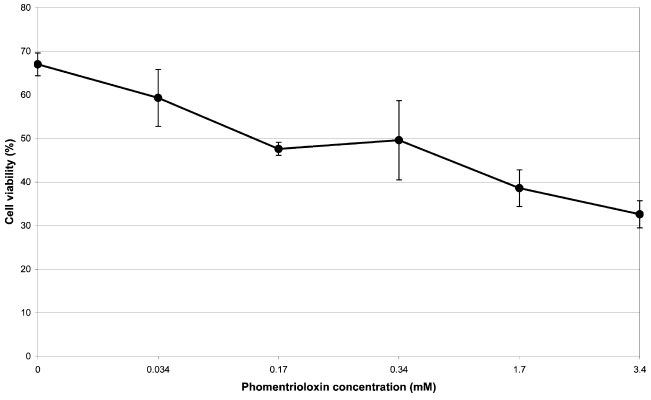

Figure 4. Effect of phomentrioloxin (**1**) on protoplasts of *Arabidopsis thaliana* (see Experimental Section for the experimental details).

of phomentrioloxin as a natural and environmentally friendly herbicide.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotation was measured on a Jasco P-1010 digital polarimeter, IR spectra were recorded as glassy film on a Perkin-Elmer Spectrum One FT-IR spectrometer, and UV spectra were recorded in MeCN solution on a Perkin-Elmer Lambda 25 UV/vis spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded at 400/100 MHz in $CDCl_3$ on Bruker spectrometers. The same solvent was used as an internal standard. Carbon multiplicities were determined by DEPT spectra.[18] DEPT, COSY-45, HSQC, HMBC, and NOESY experiments[18] were performed using Bruker microprograms. HRESI and ESIMS spectra were recorded on Waters Micromass Q-TOF Micro and Agilent Technologies 6120 Quadrupole LC/MS instruments, respectively. Analytical and preparative TLC were performed on silica gel plates (Merck, Kieselgel 60 F_{254}, 0.25); the spots were visualized by exposure to UV light and/or by spraying first with 10% H_2SO_4 in MeOH and then with 5% phosphomolybdic acid in EtOH, followed by heating at 110 °C for 10 min. CC: silica gel (Merck, Kieselgel 60, 0.063–0.200 mm).

Fungal Strain. The fungal strain of *Phomopsis* sp. used in this study was isolated from symptomatic saffron thistle (*C. lanatus*) plants in Australia in 1994.[6] The fungus was identified by Dr. Micheal Priest (New South Wales Department of Primary Industries, ASCU Orange Agricultural Orange, Australia) and deposited as DAR Herb 73822 saffron thistle (*C. lanatus*) plants in Australia in 1994.[6] Pure cultures were maintained on potato-dextrose-agar (PDA, Sigma-Aldrich Chemic GmbH, Buchs, Switzerland) and stored in the collection of the Istituto di Scienze delle Produzioni Alimentari, CNR, Italy, with the code ITEM13496.

Production, Extraction, and Purification of Phomentrioloxin (1). The fungus was grown in 1 L Erlenmeyer flasks containing 300 mL of a defined mineral.[35] Each flask was seeded with 5 mL of a mycelia suspension and then incubated at 25 °C for 4 weeks in the dark. After mycelial removal by filtration, the culture filtrates (4 L) were lyophilized and then dissolved in 400 mL of distilled H_2O, acidified to pH 2.5 with HCOOH, and extracted with EtOAc (4 × 400 L). The organic extracts were combined, dried by Na_2SO_4, and evaporated under reduced pressure, giving a brownish-red, oily residue (937 mg) showing high phytotoxic activity. It was fractionated by a silica gel column eluted with $CHCl_3$–*i*-PrOH (9:1), obtaining 11 groups of homogeneous fractions. Groups 5, 6, 9, and 10 proved to be phytotoxic and were further purified. The residues of fractions 5 and 6 were combined (170 mg) and purified by preparative TLC, eluted with $CHCl_3$–*i*-PrOH (9:1), producing four bands. The third band (81 mg) was purified under the same conditions to afford phomentrioloxin (**1**, R_f 0.32, 40.3 mg, 10.0 mg/L) as a homogeneous solid. It was crystallized by slow evaporation from a MeOH–H_2O (3:1) solution.

Phomentrioloxin (1). $[\alpha]^{25}_D$ −23 (*c* 0.4, $CHCl_3$); IR ν_{max} 3385, 2186, 1666, 1628, 1604 cm^{-1}; UV λ_{max} nm (log ε) 259 (4.25), 240 (4.25), 230 (4.25); ^{1}H and ^{13}C NMR spectra, see Table 1; ESIMS (+) *m*/*z* 607 $[2\ M + Na]^+$, 331 $[M + K]^+$, 315.1561 [calcd for $C_{17}H_{24}NaO_4$ 315.1572, $M + Na]^+$.

1,2,4-*O*,*O*′,*O*″-Triacetylphomentrioloxin (2). Phomentrioloxin (15.0 mg) was acetylated with pyridine (50 μL) and Ac_2O (50 μL) at room temperature for 10 min. The reaction was stopped by addition of MeOH, and the azeotrope, obtained by the addition of benzene, was evaporated by an N_2 stream. The oily residue (13.7 mg) was purified by preparative TLC, eluted with $CHCl_3$–*i*-PrOH (97:3), to give the 1,2,4-*O*,*O*′,*O*″-triacetyl derivative **2** of phomentrioloxin as a homogeneous compound (R_f 0.63, 3.2 mg). Derivative **2**: $[\alpha]^{25}_D$ −132 (*c* 0.3, $CHCl_3$); IR ν_{max} 2335, 1747, 1637,1599, 1220 cm^{-1}; UV λ_{max} nm (log ε) 273 (sh), 261 (4.56); ^{1}H NMR, see Table 2; ESIMS (+) *m*/*z* 441 $[M + Na]^+$.

1,2-*O*,*O*′-Isopropylidene of Phomentrioloxin (3). To phomentrioloxin (12.0 mg) dissolved in dry acetone (12.0 mL) was added anhydrous $CuSO_4$ (480.0 mg). The reaction was carried out by reflux under stirring for 2 h and then stopped by filtration. The solution obtained was evaporated under reduced pressure. The residue (13.4 mg) was purified by preparative TLC, eluted with $CHCl_3$–*i*-PrOH (97:3), yielding **3** as a homogeneous oil (R_f 0.53, 6.3 mg): $[\alpha]^{25}_D$ +21 (*c* 0.2); IR ν_{max} 3450, 2280, 1725, 1631, 1604 cm^{-1}; UV λ_{max} nm (log ε) 260 (5.01); ^{1}H NMR, see Table 2; ESIMS (+) *m*/*z* 687 $[2M + Na]^+$, 355 $[M + Na]^+$.

4-*O*-(*S*)-α-Methoxy-α-trifluoromethyl-α-phenylacetate (MTPA) Ester of Derivative 3 (4). (*R*)-(−)-MPTA-Cl (10 μL) was added to **3** (2.0 mg) dissolved in dry pyridine (20 μL). The mixture was kept at room temperature for 1 h, and then the reaction stopped

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by adding MeOH. Pyridine was removed by an N_2 stream. The residue (2.5 mg) was purified by preparative TLC, eluted with $CHCl_3$, yielding **4** as a homogeneous oil (R_f 0.69, 2.1 mg): IR ν_{max} 2389, 1750, 1451, 1381, 1244 1169 cm^{-1}; UV λ_{max} nm (log ε) 260 (4.56); ^1H NMR, see Table 2; ESIMS (+) m/z 571 $[M + Na]^+$.

4-*O*-(*R*)-α-Methoxy-α-trifluoromethyl-α-phenylacetate (MTPA) Ester of Derivative 3 (5). (*S*)-(+)-MPTA-Cl (10 μL) was added to **3** (2.0 mg) dissolved in dry pyridine (20 μL). The reaction was carried out under the same conditions used for preparing **4**. The purification of the crude residue (2.2 mg) by preparative TLC (solvent system D) gave **5** as a homogeneous oil (R_f 0.69, 1.6 mg): IR ν_{max} 2377, 1750, 1451, 1377, 1237, 1168 cm^{-1}; UV λ_{max} nm (log ε) 261 (4.85); ^1H NMR, see Table 2; ESIMS (+) m/z 571 $[M + Na]^+$.

Crystal Structure Determination of Phomentrioloxin (1). Colorless, block-shaped single crystals of **1** were obtained at ambient temperature by slow evaporation of a MeOH−H_2O (3:1) solution. X-ray data collection was performed at 173 K under N_2 flow on a Bruker-Nonius KappaCCD diffractometer equipped with graphite-monochromated Mo Kα radiation (λ = 0.71073 Å, CCD rotation images, thick slices, φ and ω scans to fill asymmetric unit). Cell parameters were obtained from a least-squares fit of the θ angles of 44 reflections in the range $3.818° \leq \theta \leq 18.589°$. A semiempirical absorption correction (multiscan, SADABS) was applied. The structure was solved by direct methods and anisotropically refined by the full matrix least-squares method on F^2 against all independent measured reflections (SIR97 package)[36] and refined by the full matrix least-squares method on F^2 against all independent measured reflections (SHELXL program of the SHELX97 package).[37] The position of hydroxy H atoms was determined from a difference Fourier map and refined according to a riding model. In the absence of significant anomalous scatters, the absolute configuration cannot be determined. Friedel pairs were therefore merged before the final refinement. The final refinement converged to R1 = 0.0546 for 1627 observed reflections having $I > 2\sigma(I)$. Minimum and maximum residual electronic density was −0.211 and 0.236 e $Å^{-3}$. Crystal data: formula $C_{17}H_{24}O_4$, formula weight 292.36 g mol^{-1}, orthorhombic $P2_12_12_1$, a = 4.632(3) Å, b = 11.982(7) Å, c = 29.48(2) Å, $\alpha = \beta = \gamma = 90°$, 6405 collected reflections, 1627 unique reflections.

Phytotoxic Activity. (**a**) For the bioassay-guided purification of the phytotoxic compounds, culture filtrates, organic extracts, and chromatographic fractions were assayed by using a leaf puncture assay as described below. Extracts and fractions were dissolved in MeOH (20 μg extract/μL) and then diluted with distilled H_2O (final concentration of MeOH = 2%). Phomentrioloxin (**1**) was tested at concentrations of 6.85, 3.42, and 1.71 mM, by applying 20 μL of solution to detached leaves previously punctured with a needle. It was tested against *Carthamus lanatus* (the host plant of the pathogen, family Asteraceae), *Mercurialis annua* L. (Euphorbiaceae), *Chenopodium album* L. (Chenopodiaceae), *Cirsium arvense* (L.) Scop. (Asteraceae), *Sonchus oleraceus* L. (Asteraceae), and *Setaria viridis* (L.) P. Beauv (Poaceae). Five replications (droplets) on separate leaves were used for each metabolite and for each plant species tested. Leaves were kept in a moistened chamber under continuous fluorescent lights. Symptoms were estimated five days after droplet application, by using a visual empiric scale from 0 (no symptoms) to 4 (wide necrosis, around 1 cm diameter). The two derivatives were assayed by using the same protocol.

(**b**) Pure compounds were tested against *Lemna minor* at concentrations between 6.85 mM and 68.5 μM, by adapting a protocol already described.[38] Briefly, the wells of sterile, polystyrene 96-well microtiter plates were filled with a 50 μL aliquot of solutions containing the metabolites to be tested, at the concentration reported above. One frond of actively growing axenic *L. minor* was placed into each well. Control wells were included in each plate. Four replications were prepared for each compound. The plates were incubated in a growth chamber with 12/24 h fluorescent lights and observed daily up to 4 days. One day after the application of the test solution, 100 μL of distilled H_2O was added to each well. The appearance of necrosis or chlorosis was assessed visually by comparison of the treated plants with the control appearance. Moreover, plantlet fresh weight was measured, and chlorophyll contents were determined by using the protocol described.[39]

(**c**) For the assay on protoplasts, callus cultures of *Arabidopsis thaliana* L. Heynh. Ecotype Landsberg (kindly provided by S. Caretto and G. Colella, ISPA CNR Lecce) were maintained at 25 °C under continuous fluorescent white light on MS (Sigma) basal medium supplemented with 30 g/L sucrose, 0.5 mg/L naphthaleneacetic acid, 0.05 mg/L kinetin, pH 5.5, and 0.6% (w/v) agar. The callus cultures were subcultured at 4-week intervals by transferring approximately 50 mg of callus tissue in Magenta boxes containing 100 mL of the same agar medium. Extraction and assay on protoplasts were performed according to the protocol described by Zonno et al. (2008).[40] Four replications were prepared for each concentration. The experiment was repeated twice. The number of viable cells was expressed as a percentage of the total number of cells.

The bioassay for the inhibition of rootlet elongation was carried out on tomato (*Lycopersicon esculentum*) seeds (var. Marmande). Briefly, seeds were surface sterilized by NaOCl (1%), washed, and allowed to germinate in Petri dishes. Ten germinated seeds were then transferred to small plates containing 1 mL of the test solution (1.37 mM). Plates were kept in a incubator at 25 °C in the dark for 4 days and then rootlets measured. Their length was compared to that of the controls. Three replications were prepared for each compound tested. The experiment was repeated twice.

Antimicrobial Activity. The antifungal activity of phomentrioloxin and the two derivatives was tested on *Geotrichum candidum*, whereas the antibacterial activity was assayed against *Bacillus subtilis* (Gram +) and *Escherichia coli* (Gram −), according to the protocols already described, by using up to 100 μg of each metabolite/diskette.[41] Three replications were performed for each compound. The zootoxic activity of the three metabolites was tested on larvae of *Artemia salina* L. (brine shrimp) up to 1.71×10^{-4} M, as previously described,[41] with four replications.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Spectra of **1** and a cif data file are available free of charge via the Internet at http://pubs.acs.org. Crystallographic data for the structure have also been deposited with the Cambridge Crystallographic Data Centre as supplementary publication number CCDC 872237. These data can be obtained free of charge at www.ccdc.cam.ac.uk/conts/retrieving.html or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: (internat.) +44-1223/336-033.

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Notes

The authors declare no competing financial interest.

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