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Phyllostictines A–D, oxazatricycloalkenones produced by *Phyllosticta cirsii*, a potential mycoherbicide for *Cirsium arvense* biocontrol

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Abstract

Phyllosticta cirsii, a fungal pathogen isolated from *Cirsium arvense* and proposed as biocontrol agent of this noxious perennial weed, produces in liquid cultures different phytotoxic metabolites with potential herbicidal activity. Four new oxazatricycloalkenones, named phyllostictines A–D, were isolated and characterized using essentially spectroscopic and chemical methods. Tested by leaf-puncture assay on the fungal host plant phyllostictine A proved to be highly toxic. The phytotoxicity decreases when both the dimension and the conformational freedom of the macrocyclic ring change, as in phyllostictines B and D, and it is totally lost when also the functionalization of the same ring is modified, as in phyllostictine C. Beside its phytotoxic properties, phyllostictine A has no antifungal activity, an interesting antibiotic activity only against Gram+ bacteria, and a noticeable zootoxic activity when tested at high concentrations. The integrity of the oxazatricycloalkenone system appears to be an important feature to preserve these activities.

Keywords: *Cirsium arvense*; *Phyllosticta cirsii*; Phytotoxins; Oxazatricycloalkenones; Phyllostictines A–D; Bioherbicides

1. Introduction

Cirsium arvense (L.) Scop. (commonly called Canada thistle) is a persistent perennial weed that grows vigorously, forming dense colonies, and spreading by roots growing horizontally that give rise to aerial shoots. It spreads by seed either by wind or as a contaminant in crop seed. Canada thistle is native to south eastern Europe and the eastern Mediterranean area. It has spread to most temperate parts of the world and is considered an important weed all around the world as it infests many habitats such as cultivated fields, roadsides, pastures and rangeland, railway embankments, and lawns. There is no easy method of control, and all methods require follow-up. Combinations of mechanical, cultural, and chemical methods are more effective than any single method used alone.[1] Although there are no effective biological control organisms available at this time, search for efficacious biocontrol microorganisms and natural herbicides is receiving a renewed interest.

Recently, the fungus *Phyllosticta cirsii* has been evaluated as a possible biocontrol agent of Canada thistle.[2] Species belonging to the genus *Phyllosticta* are known to produce bioactive metabolites, including non-host phytotoxins, e.g., phyllosinol, brefeldin, and PM-toxin isolated by cultures of *Phyllosticta* sp.,[3] *Phyllosticta maydis*,[4] and *Phyllosticta medicaginis*,[5] respectively.

Considering the interest for bioactive metabolites produced by weed pathogens as sources of novel natural herbicides, it

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seemed interesting to investigate the production of toxins by this species of *Phyllosticta*.

This paper describes the isolation, structural elucidation, and biological characterization of four new phytotoxic oxazatricycloalkenones produced in liquid culture by *P. cirsii*, named phyllostictines A–D (**1**–**4**) (Fig. 1). Their structure was determined by extensive use of spectroscopic (essentially NMR and MS techniques) and chemical methods.

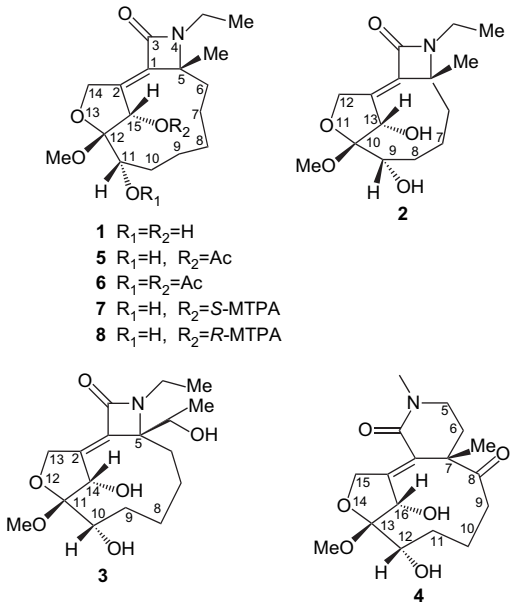

Figure 1. Structures of phyllostictine A and its derivatives (**1** and **5**–**8**) and of phyllostictines B–D (**2**–**4**).

2. Results and discussion

The liquid culture of *P. cirsii* (7.7 L) was exhaustively extracted as reported in Section 4. The organic extract, having high phytotoxicity, was purified by a combination of CC and TLC as described in Section 4. Four metabolites were obtained as homogeneous oily compounds (11.0, 1.0, 0.9, and 0.5 mg/L, respectively), which were named phyllostictines A–D (**1**–**4**). Preliminary ^{1}H and ^{13}C investigations allowed to demonstrate that these metabolites have closely related structures being, as described below, four novel oxazatricycloalkenones.

Phyllostictine A (**1**) is the main phytotoxic metabolite and has a molecular formula $C_{17}H_{27}NO_5$, as deduced from HRESIMS spectra, consistent with five unsaturations. Two of them were a tetrasubstituted double bond and a carbonyl lactam group, as deduced from the IR spectrum and preliminary ^{1}H and ^{13}C NMR investigations. The IR spectrum also showed bands attributable to hydroxy groups,[6] while the UV spectrum exhibited an absorption maximum typical of α,β-unsaturated lactams.[7] In particular, the ^{1}H NMR spectrum (Table 1) of phyllostictine A showed the presence of one broad and two sharp singlets at δ 4.45, 3.91, and 1.27, respectively, attributable to the protons of a secondary hydroxylated carbon (HO–CH-15), to a methoxy, and to a tertiary methyl (Me–C-5) group, respectively.[8] H-15 appeared long-range coupled ($J<1$ Hz) in the COSY spectrum[9] with the broad singlet of a hydroxy group resonating at δ 3.61. A broad singlet attributable to another hydroxy group was also observed at δ 2.80. In the same spectrum, the significant presence of two sharp doublets ($J=0.9$ Hz), typical of an AB system of an oxygenated methylene group (H_2C-14), as well as, a multiplet due to a proton of another hydroxylated secondary carbon (HO–CH-11) were observed at δ 5.08 and 5.03, and 4.04, respectively. In the COSY spectrum the latter coupled with one or both the protons of the adjacent methylene group (H_2C-10) resonating as complex multiplet at δ 1.80, which were, in turn, coupled with the protons of the successive methylene group (H_2C-9) observed as multiplets at δ 1.58 and 1.37, respectively. The region of the aliphatic protons presented also a very complex multiplet at δ 1.30–1.26 attributable to the protons of three other methylene groups (H_2C-8, H_2C-7, and H_2C-6),[8] which are coupled themselves and with the protons of H_2C-9, as appeared from the COSY spectrum. Furthermore, the triplet ($J=7.1$ Hz) of the methyl (*Me*CH_2–N) of an *N*-ethyl group resonated at δ 0.83 and coupled with the protons of the adjacent methylene group (Me*C*H_2–N) in the COSY spectrum, which overlapped at δ 1.30 with the complex signals of the above-described methylene groups (H_2C-8, H_2C-7, and H_2C-6).[8] The ^{13}C NMR spectrum (Table 2) showed the presence of the signals of a lactam carbonyl and those of the α,β-conjugated tetrasubstituted double bond at the typical chemical shift values of δ 166.6, 156.2, and 136.3 (C-3, C-2, and C-1), respectively.[10] The oxygenated methylene and two hydroxylated methyne carbons observed at δ 92.7, 86.3, and 68.4 were attributed, also based on the coupling observed in the HSQC spectrum,[9] to C-14, C-11, and C-15, respectively. Similarly, the signals at δ 64.5, 17.1, and 14.1 were assigned to the methoxy, the tertiary methyl (Me–C-5), and the methyl group of the *N*-ethyl residue and those at δ 27.5 and 26.5 to the methylene carbons C-10 and C-9.[10] Finally, the two signals at δ 104.3 and 71.8 were assigned to the dioxygenated and nitrogen linked quaternary carbons C-12 and C-5.[10] The latter represents the closure of the 3,5-dihydroxy-4-methoxy-11-methylcycloundec-1-ene macrocyclic ring. This hypothesis was confirmed by the typical chemical shift values of δ 29.7, 29.3, and 22.6 (C-8, C-7, and C-6, respectively) observed for the carbons of the other three methylene groups belonging to the macrocyclic ring[10] and the couplings observed in the HMBC spectrum[9] (Table 3). In fact, in the HMBC spectrum, C-5 coupled with H_2-6, Me–C(5), and long range with H-15. The correlations observed in this latter spectrum also allowed to locate the tertiary methyl group on C-5, which represents one of the bridgehead carbons of the junction between the macrocyclic and the *N*-ethyl β-lactam (2-azetidone) rings, while the other one is the olefinic carbon C-1. Based also on the coupling observed in the HMBC spectrum (Table 3), the remaining unsaturation was attributed to a 2,2,3,4-tetrasubstituted 2,3,5-trihydrofuran ring, which was joined with the macrocyclic ring through two bridgehead carbons, namely the other quaternary olefinic (C-2) and the dioxygenated quaternary (C-12) carbons. In fact, in HMBC spectrum, C-1 coupled with H_2-14 and C-2 with these latter protons, H-15, and long range with H-11, while C-12 coupled H-15. On the basis of these

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

Position	δ_H			
	Compound **1**	Compound **2**	Compound **3**	Compound **4**
5				2.44 (2H) t (J=7.3 Hz)
6	1.30 (2H) m	1.33 (2H) m	1.34 (2H) m	1.61 m, 1.36 m
7	1.30 (2H) m	1.58 m, 1.38 m	1.40 (2H) m	
8	1.30 m, 1.26 m	1.80 (2H) m	1.60 m, 1.38 m	
9	1.58 m, 1.37 m	4.02 m	1.80 (2H) m	1.36 (2H) m
10	1.80 (2H) m		4.03 m	1.61 m, 1.40 m
11	4.04 m			1.81 (2H) m
12		5.08 d (J=1.0 Hz), 5.04 d (J=1.0 Hz)		4.02 m
13		4.48 br s	5.07 d (J=1.0 Hz), 5.05 d (J=1.0 Hz)	
14	5.08 d (J=0.9 Hz), 5.03 d (J=0.9 Hz)		4.46 br s	
15	4.45 br s			5.06 d (J=1.3 Hz), 5.04 d (J=1.3 Hz)
16				4.47 br s
MeN				2.14 s
Me*CH*$_2$N	1.30 (2H) m	1.33 (2H) m	1.48 (2H) m	
*Me*CH$_2$N	0.83 t (J=7.1 Hz)	0.91 t (J=6.6 Hz)	0.95 t (J=7.1 Hz)	
Me–C(5)	1.27 s	1.26 s		
Me*CH*(OH)–C(5)			3.80 m	
*Me*CH(OH)–C(5)			1.18 d (J=6.2 Hz)	
Me–C(7)				1.26 s
MeO	3.91 s	3.92 s	3.91 s	3.92 s
OH	3.61 (br s), 2.80 (br s)	3.10 (br s), 2.20 (br s)	3.29, 2.45, 1.61 (all br s)	3.06 (br s), 2.24 (br s)

[a] The 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.

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

Position	δ_C m[c]			
	Compound **1**	Compound **2**	Compound **3**	Compound **4**
1	136.3 s	136.3 s	136.4 s	136.4 s
2	156.2 s	156.0 s	156.0 s	155.9 s
3	166.6 s	166.2 s	166.4 s	166.3 s
5	71.8 s	71.8 s	71.8 s	43.6
6	22.6 t	22.6	25.6 t	23.6 t
7	29.3 t	26.2 t	29.2 t	71.8 t
8	29.7 t	27.5 t	26.4 t	210.0 s
9	26.5 t	86.18 d	27.4 t	28.8 t
10	27.5 t	104.2 s	86.2 d	26.3 t
11	86.3 d			27.3 t
12	104.3 s	96.2 t		86.0 d
13		68.9 t	92.6 t	104.4 s
14	92.7 t		68.6 d	
15	68.4 d			92.6 t
16				68.1 d
Me*CH*$_2$N	31.8 t	31.6 t	39.2 t	
*Me*CH$_2$N	14.1 q	14.1 q	14.1 q	
MeN				29.9 q
Me–C(5)	17.1 q	17.1 q		16.5 q
Me*CH*(OH)–C(5)			68.1 d	
*Me*CH(OH)–C(5)			23.6 q	
Me–C(7)				
MeO	64.5 q	64.6 q	64.6 q	64.6 q

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

results phyllostictine A appears to be a new oxazatricycloalkenone, to which the structure of a 4-ethyl-11,15-dihydroxy-12-methoxy-5-methyl-13-oxa-4-aza-tricyclo[10.2.1.0*2,5*]-pentadec-1-en-3-one (**1**) can be assigned. This structure was confirmed by the results observed in the ESI and EI mass spectra. In fact, the HRESIMS spectrum, recorded in positive modality, showed sodium clusters formed by the toxin itself and the corresponding dimer and trimer at *m/z* 348.1800, $[M+Na]^+$ 673.3680 $[2M+Na]^+$ and 998 $[3M+Na]^+$, respectively, as well as the pseudomolecular ion $[M+H]^+$ at *m/z* 326.1962. The same spectrum, recorded in negative modality, showed the pseudomolecular ion $[M-H]^-$ and that of the corresponding dimer $[2M-H]^-$ at *m/z* 324.1815 and 649.3678, respectively. Significant were the data of the EIMS spectrum, which did not show the molecular ion but peaks due to fragmentation typical of the presence both a β-lactam and a suitable substituted trihydrofuran ring, methoxy, hydroxy, and tertiary methyl groups.[8,11] In fact, the molecular ion losing in succession the methoxy group and H_2O generated the ions at *m/z* 294 and 276. Alternatively, the molecular ion losing in succession the methoxy group followed by CO and Me residues yielded the ion at *m/z* 251. Significant for the presence of the β-lactam residue is the most abundant ion $[Et-N{=}C{=}O]^+$ observed at *m/z* 71.[11]

The structure assigned to phyllostictine A was further supported by converting the toxin into the mono- and diacetyl derivatives (**5** and **6**) by the usual reaction with pyridine and acetic anhydride. The spectroscopic data of both derivatives

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

C	HMBC			
	Compound **1**	Compound **2**	Compound **3**	Compound **4**
1	H_2-14	H_2-12	H_2-13	H_2-15
2	H-15, H_2-14, H-11	H-13, H_2-12, H-9	H-14, H_2-13	H-16, H_2-15, MeO
5	H-15, H_2-6, Me–C(5)	H-13, Me–C(5)	H-14, H_2-6, Me*C*H_2N	H_2-6, MeN
6	Me–C(5)	Me*C*H_2N, H-9, H-7′		H_2-5, MeN
7		Me–C(5)		H_2-6, Me–C(7)
8		H-9		H_2-6, H_2-5
9	H_2-10	H_2-8	H-10	
10	H-11, H_2-9	H-13	H_2-9, H_2-8	
11	H-15, H_2-10, H_2-9		H-14	
12	H-15			H_2-11, H_2-10
13				H-16
MeN				H_2-6, H_2-5
Me*C*H_2N	*Me*CH_2N	*Me*CH_2N	*Me*CH_2N	
Me*C*H(OH)–C(5)			*Me*CH(OH)–C(5)	

were fully consistent with the structure **1** assigned to the toxin. In particular, the IR spectrum of 15-*O*-acetylphyllostictine A (**5**) still showed the presence of hydroxy groups, which are obviously absent in the 11,15-diaceyl derivative (**6**). The ^{1}H and ^{13}C NMR spectra of **5** differed from those of **1** for the significant downfield shift ($\Delta\delta$ 1.14) of H-15 at δ 5.59 and for the presence of the singlet of the acetyl group at δ 2.19, respectively,[8] and for the presence of the signals of the acetyl group observed at δ 172.4 (Me*C*O) and 20.9 (*Me*CO).[10] Similarly, the same spectra of **6**, compared to those of **1**, showed, respectively, the downfield shift of both H-15 and H-11 ($\Delta\delta$ 1.14 and 1.16) at δ 5.59 and 5.20 and the presence of the singlets of two acetyl groups at δ 2.13 and 1.99,[8] and the signals of the two acetyl groups at δ 170.1 and 169.9 (two Me*C*O) and δ 22.1 and 20.8 (two *Me*CO).[10]

The other three phyllostictines B–D (**2**–**4**) appear to be very closely related to phyllostictine A and each other.

Phyllostictine B (**2**) has a molecular formula of $C_{15}H_{23}NO_5$ as deduced from HRESIMS spectra consistent with the same five unsaturations of **1**, which are in agreement with the IR bands and the preliminary ^{1}H and ^{13}C NMR investigations, but differed for the lack of two CH_2 groups. As expected, the IR and UV spectra were very similar to those of **1**. The investigation of the ^{1}H and ^{13}C NMR spectra (Tables 1 and 2) confirmed that the two toxins differed in the size of the macrocyclic ring, which is 3,5-dihydroxy-4-methoxy-11-methylcycloundec-1-ene in **1**, while it is 3,5-dihydroxy-4-methoxy-9-methyl-cyclonon-1-ene in **2**. The couplings observed in the COSY and HSQC spectra allowed to assign the chemical shifts to all the protons and the corresponding carbons (Tables 1 and 2, respectively) and to phyllostictine B the structure of 4-ethyl-9,13-dihydroxy-10-methoxy-5-methyl-11-oxa-4-aza-tricyclo[8.2.1.0*2,5*]tridec-1-en-3-one (**2**). This structure was supported by the several couplings observed in the HMBC spectrum (Table 3). Significant were the couplings observed between C-1 and H_2-12 and C-2 with these latter protons, H-13 and long range with H-9, as well as those of C-5 with Me–C(5) and long range with H-13, and in that of this latter proton with C-10. The structure was further confirmed by the pseudomolecular ion and sodium clusters observed in the HRESIMS spectrum for the toxin itself and its dimer and trimer at *m/z* 298.1628 $[M+H]^+$ and 320.1443 $[M+Na]^+$, 617.2984 $[2M+Na]^+$, 914 $[3M+Na]^+$, respectively. Furthermore, the same spectrum recorded in negative modality showed the pseudomolecular ion $[M-H]^-$ at *m/z* 296.1500. The structure **2** was further supported by the data of its EIMS spectrum, which showed, beside the pseudomolecular ion $[MH]^+$ at *m/z* 298, ions produced by fragmentation mechanisms similar to those observed in **1**. In fact, the pseudomolecular ion by loss of H_2O generated the ion at *m/z* 280, as well as the molecular ion $[M]^+$ produced the ions at *m/z* 266, 248, and 223 by successive loss of methoxy, H_2O, and Me residues, respectively. Finally, the most abundant ion $[Et-N{=}C{=}O]^+$, which is significantly due to the presence of the β-lactam residue, was observed at *m/z* 71.[8,11]

Phyllostictine C (**3**) has a molecular formula of $C_{17}H_{27}NO_6$ as deduced from HRESIMS spectrum consistent with the same five unsaturations of **1**, which are in agreement with the IR bands and the preliminary ^{1}H and ^{13}C NMR investigations. A comparison of both ^{1}H and ^{13}C NMR spectra of phyllostictine C with those of **1** showed that the two toxins differed in the substituent at C-5 and in the macrocyclic ring size, which in **3** is 3,5-dihydroxy-4-methoxy-10-(1-hyroxyethyl)-cyclodec-1-ene. In fact, in the region of the aliphatic methylene group of both spectra of **3** signals accounting for only four methylene protons are present, and lacked the signal of the tertiary methyl group, which in **1** is linked to C-5. On the contrary, the significant presence of the signals of 1-hydroxyethyl group was observed.[8,10] In particular, the ^{1}H NMR spectrum showed the presence of the multiplet due to the proton of a further secondary hydroxylated carbon (Me*CH*–OH), the doublet (J=6.2 Hz) of the adjacent terminal methyl group (*Me*–CH–OH), and the broad singlet of a further hydroxy group at δ 3.80, 1.18, and 1.61, respectively.[8] The ^{13}C NMR spectrum showed the signals of the corresponding secondary hydroxylated carbon (Me*CH*–OH) and methyl group (*Me*CH–OH) at δ 68.1 and 23.6.[10] The couplings observed in the COSY and HSQC spectra allowed to assign the chemical shifts

to all the protons and the corresponding carbons (Tables 1 and 2, respectively) and to phyllostictine C the structure of 4-ethyl-10,14-dihydroxy-5-(1-hydroxyethyl)-11-methoxy-12-oxa-4-aza-tricyclo[9.2.1.0*2,5*]tetradec-1-en-3-one (**3**). This structure was supported by the several couplings observed in the HMBC spectrum (Table 3). Significant were the couplings observed between C-1 and H_2-13 and C-2 with these latter protons and H-14, as well as those of C-5 with H_2-6 and long range with H-14, and that of this latter proton with C-1. The structure was also confirmed by the sodium clusters observed in the HRESIMS spectrum for the toxin itself and its dimer and trimer at *m/z* 364.1707 $[M+Na]^+$, 705 $[2M+Na]^+$, and 1046 $[3M+Na]^+$, respectively.

Phyllostictine D (**4**) has a molecular formula of $C_{17}H_{25}NO_6$ as deduced from HRESIMS spectrum consistent with five unsaturations, four of which were the same observed as in **1** and in agreement with the IR bands and preliminary ^1H and ^{13}C NMR investigations. A comparison of both ^1H and ^{13}C NMR spectra of phyllostictine D with those of **1** showed that the two toxins differed in the size and the functionalization of both the lactam and macrocyclic rings. In **4** the lactam appears to be an *N*-methyl-δ-lactam (2-piperidone) always joined, through the same two bridgehead carbons to the macrocyclic ring, which in **4** is 3,5-dihydroxy-4-methoxy-10-methyl-9-oxo-cyclodec-1-ene. In fact, the region of the aliphatic methylene and methyl groups of both ^1H and ^{13}C NMR spectra of **4**, compared to those of **1**, showed substantial differences. Complex multiplets and one singlet accounting for only three methylene groups belong to the macrocyclic ring and the methyl group bonded to the bridgehead quaternary carbon (C-7) was observed in the ^1H NMR spectrum. In addition, the triplet (J=7.3 Hz) and the singlet of a methylene (CH_2-5) and methyl (N−Me) bonded to a nitrogen atom were observed at the typical chemical shift values of δ 2.44 and 2.14, respectively,[8] while the protons of the other methylene (CH_2-6) group of the δ-lactam ring adjacent to CH_2-5 resonated as two complex multiplets at δ 1.61 and 1.36. In the ^{13}C NMR spectrum the carbons of these two methylene groups and that of *N*-methyl group appeared at the very typical chemical shift values of δ 43.6, 23.6, and 29.9 (C-5, C-6, and Me−N), respectively.[10] In addition, the signal of a saturated ketone group (O=C-8) was observed at the expected chemical shift value of δ 210.0.[10] The couplings observed in the COSY and HSQC spectra allowed to assign the chemical shifts to all the protons and the corresponding carbons (Tables 1 and 2, respectively) and to phyllostictine D the structure of 12,16-dihydroxy-13-methoxy-4,7-dimethyl-14-oxa-4-aza-tricyclo[11.2.1.0*2,7*]hexadec-1-en-3,8-dione (**4**). This structure was supported by the several couplings observed in the HMBC spectrum (Table 3). Significant were the couplings observed between C-1 and H_2-15 and C-2 with these latter protons and H-16, as well as those of C-5 with H_2-6 and N−Me, C-6 with H_2-5 and Me−N, C-7 with H_2-6 and Me−C(7), C-8 with H_2-6 and long range with H_2-5, and finally C-13 with H-16. The structure was also confirmed by the sodium clusters observed in the HRESIMS spectrum for the toxin itself and its dimer and trimer at *m/z* 362.1548 $[M+Na]^+$, 701 $[2M+Na]^+$, and 1040 $[3M+Na]^+$, respectively.

2.1. Absolute stereochemistry of phyllostictines A–D (1–4)

The absolute stereochemistry of the secondary hydroxylated carbon C-15 of phyllostictine A (**1**) was determined by applying Mosher's method.[12–14] By reaction with *R*-(−)-α-methoxy-α-trifluorophenylacetate (MTPA) and *S*-(+)MTPA chlorides, phyllostictine A was converted into the corresponding diastereomeric *S*-MTPA and *R*-MTPA esters (**7** and **8**, respectively), whose spectroscopic data were consistent with the structure assigned to **1**. The comparison between the ^1H NMR data (see Section 4) of the *S*-MTPA ester (**7**) and those of the *R*-MTPA ester (**8**) of **1** [$\Delta\delta$ (**7**−**8**): H-11 +0.07; H_2-10 +0.17; H-9 +0.24; H-9′ +0.13] allowed to assign a *S*-configuration at C-15. The significant effects observed in the NOESY spectrum (Table 4) between H-15 with H-11, MeO, and Me−C(5) and between MeO and H-11 allowed to assign the relative configuration to C-15, C-12, C-11, and C-5 being the H-15, MeO, H-11, and Me−C(5) at the same side of the molecule, and consequently to the double bond an *E*-configuration.[8] Considering the absolute *S*-stereochemistry determined for C-15, the absolute stereochemistry of C-5, C-11, and C-12 should be *R*, *S*, and *S*, respectively.

On the basis of the similar spectroscopic properties of phyllostictines B−D with those of phyllostictine A and the NOESY effects recorded for these toxins (Table 4), the absolute stereochemistry of the chiral centers of **2**−**4** could be assigned as that observed in **1** and as depicted in their structural formulae with the exception of C-7 of **4**, which should be *S* as the substituent priority is opposite in respect to that of **1**.

Table 4
2D ^1H NOE (NOESY) data obtained for phyllostictines A−D (**1**−**4**)

1		**2**		**3**		**4**	
Considered	Effects	Considered	Effects	Considered	Effects	Considered	Effects
H-15	H_2-14, H-11, MeO, Me−C(5)	H-13	H_2-12, H-9, MeO, OH, OH, H-7, Me−C(5)	H-14	H_2-13, H-10, MeO, OH, OH, *Me*CH(OH)−C(5)	H-16	H_2-15, H-12, MeO, OH, OH, H_2-5, Me−C(7)
H-11	H-15, MeO, H_2-10, H-9′	H-9	H-13, MeO, OH, H_2-8, H_2-7	H-10	H-14, MeO, H_2-9, H_2-8	H-12	H-16, MeO, H_2-5, OH, H_2-11, H_2-10, Me−C(7)
MeO	H-15, H_2-14, H-11, OH, OH, Me−C(5)	MeO	H_2-12, H-13, H-9, OH, H_2-8, H-7	MeO	H-14, H_2-13, H-10, OH	MeO	H_2-15, H-16, H-12, H-10, Me−C(7)
				*Me*CH(OH)−C(5)	*Me*CH_2N	H_2-5	MeN

2.2. Biological activity

When tested at concentration around 6×10^{-3} M by the leaf-puncture assay on Canada thistle, phyllostictines had different toxicities. Phyllostictine A was particularly active, causing the fast appearance of large necrotic spots (about 6–7 mm of diameter). Phyllostictines B and D were slightly less toxic compared to the main metabolite, whereas phyllostictine C was almost not toxic (Table 5). These results showed a clear structure–activity relationship between the phytotoxic activity and the structural feature characterizing the phyllostictine group. In fact, the most toxic compound appeared to be phyllostictine A (**1**) in which the 3,5-dihydroxy-4-methoxy-11-methylcycloundec-1-ene macrocyclic ring is joined with both the *N*-ethyl β-lactam and the 2,2,3,4-tetrasubstituted-2,3,5-trihydrofuran rings. The phytotoxicity decreases in phyllostictines B and D, in which the dimension and the conformational freedom of the macrocyclic ring are changed but its functionalities remain unaltered. When also this latter changes in combination with the size and the conformational freedom, as in phyllostictine C (**3**), which showed a higher steric hindered 1-hydroethyl group at C-5 instead of the methyl group as in **1**, the toxicity was completely lost. The *N*-ethyl β-lactam ring appears to be less important for the activity as phyllostictine D (**4**), in which it became an *N*-methyl δ-lactam, showed the same level of toxicity of **2**. The importance of the 2,2,3,4-tetrasubstituted-2,3,5-trihydrofuran rings remains to be ascertained by assaying derivatives showing modifications of this moiety prepared from phyllostictine A.

Table 5
Effect of phyllostictines A–D (**1**–**4**) in the puncture assay on thistle leaves

Phyllostictine	Toxicity (20 μl/droplet)
A	++++[a]
B	+++
C	–
D	+++

[a] Toxicity is determined using the following scale: –, no toxic; +++, necrosis 3–5 mm; ++++, wider necrosis.

The antimicrobial and the zootoxic activities were assayed only for phyllostictines A and B, being phyllostictines C and D isolated in very low amounts.

In the antifungal assay on *Geotrichum candidum*, phyllostictines A and B were completely inactive assayed up to 100 μg/disk. Assayed against bacteria, only phyllostictine A was active against *Lactobacillus* sp. (Gram+) species, already at 5 μg/disk, whereas both compounds were completely inactive against *Escherichia coli* (Gram–) even when tested up to 100 μg/disk.

When tested on brine shrimp (*Artemia salina* L.) larvae only phyllostictine A caused the total larval mortality when assayed at 10^{-3} M, and a still noticeable mortality at 10^{-4} M (24%), whereas phyllostictine B proved to have a negligible activity.

In both antimicrobial and zootoxic activities, the integrity of the oxazatricycloalkenone system present in phyllostictine A appears as an important feature to preserve the activity.

3. Conclusion

Phyllostictines A–D are the first four fungal metabolites described to belong to a oxazatricycloalkenone group and to occur for the first time as natural compounds with interesting biological activity. In particular, the main fungal metabolite phyllostictine A showed potentially strong herbicidal properties not associated to antifungal or zootoxic activities, while a selective antibiosis was exhibited against Gram+ bacteria. Compounds containing macrocyclic rings as well as furan derivatives are quite common as naturally occurring compounds and some of them are biologically active,[15,16] while compounds containing β-lactam (2-azetidone) are only known as synthetic substances and some of them have pharmacological applications as hypocholesterolemic agents.[17,18] 2-Piperidones are known as naturally occurring compounds and essentially as metabolites of plants[19] and animals.[20]

4. Experimental section

4.1. General

Optical rotation was measured in $CHCl_3$ solution on a Jasco P-1010 digital polarimeter and the CD spectrum was recorded on a JASCO J-710 spectropolarimeter in $CHCl_3$ solution. IR spectra were recorded as neat 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 and 75 MHz, respectively, in $CDCl_3$ by Bruker spectrometers. The same solvent was used as internal standard. Carbon multiplicities were determined by DEPT spectra.[9] DEPT, COSY-45, HSQC, HMBC, and NOESY experiments[9] were performed using Bruker microprograms. ESI and HRESIMS spectra were recorded on Waters Micromass Q-TOF Micro and Agilent 1100 coupled to JOEL AccuTOF (JMS-T100LC) spectrometers. EIMS spectra were taken at 70 eV on a QP 5050 Shimadzu 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 (9:1); (B) $CHCl_3$–*i*-PrOH (96:4); (C) EtOEt–AcOEt (9:1); (D) EtOH–H_2O (6:4).

4.2. Fungus

P. cirsii Desm., isolated from diseased leaves of *C. arvense*, was supplied by Dr. Alexander Berestetskyi, All-Russian Research Institute of Plant Protection, Pushkin, Saint-Petersburg, Russia. The strain was maintained in sterile tubes containing potato–sucrose–agar (PDA) and subcultured when needed.

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

For the production of phytotoxic metabolites, Roux bottles (1 L) containing a mineral-defined medium (200 mL)[21] were seeded with mycelial fragments obtained from colonies actively growing on PDA plates. The cultures were incubated under static conditions at 25 °C in the dark for four weeks, then filtered on filter paper (Whatman no. 4), assayed for phytotoxic activity, and lyophilized. The lyophilized material obtained from the culture filtrates (7.7 L) was dissolved in distilled water (700 mL, final pH 4.4) and extracted with EtOAc (3×700 mL). The organic extracts were combined, dehydrated with Na_2SO_4, filtered, and evaporated under reduced pressure. The brown oily residue (1.26 g) proved to be highly phytotoxic when assayed as below described on detached thistle leaves. It was purified by silica gel column eluted with the solvent system A, and nine groups of homogeneous fractions were obtained. All the fractions were tested for their phytotoxic activity. The residue (74.0 mg) of the second fraction, which proved to be highly phytotoxic, was further purified by column chromatography eluted with the solvent system B, yielding 10 groups of homogeneous fractions. The residue of the toxic fifth fraction (359 mg) was purified by preparative TLC on silica gel (eluent C) yielding a fraction (R_f 0.38), which proved to be a mixture of at least two metabolites. It was further purified by preparative TLC on reversed phase (eluent D) yielding the main toxin and other metabolites named phyllostictines A and B (**1** and **2**), both as homogeneous oily compounds (R_f 0.36 and R_f 0.58, eluent D, 85 and 7.7 mg, 11.0 and 1.0 mg/L, respectively). The residue of the sixth fraction of the first column (74.7 mg) was further purified by preparative TLC on silica gel (eluent C) yielding a homogeneous oily compound (R_f 0.13, eluent C, 3.6 mg, 0.5 mg/L) named phyllostictine D (**4**). Finally, the residue of the eight fraction of the first column (25.9 mg) was further purified by a preparative TLC on silica gel (eluent A) yielding a homogeneous oily compound (R_f 0.32, eluent A, 6.6 mg, 0.9 mg/L) named phyllostictine C (**3**).

4.4. Phyllostictine A (**1**)

Compound **1**: $[\alpha]_D^{25}$ −87.5 (*c* 0.2); IR ν_{max} 3394, 1704, 1632, 1440 cm^{-1}; UV λ_{max} nm (log ε) 263 (4.07); ^1H and ^{13}C NMR spectra: see Tables 1 and 2; HRESIMS (+) *m/z*: 998 $[3M+Na]^+$; 673.3680 [$C_{34}H_{54}N_2NaO_{10}$, calcd 673.3677] $[2M+Na]^+$, 348.1800 [$C_{17}H_{27}NNaO_5$, calcd 348.1787] $[M+Na]^+$, 326.1962 [$C_{17}H_{28}NO_5$, calcd 326.1967] $[M+H]^+$; HRESIMS (−) *m/z*: 324.1815 [$C_{17}H_{26}NO_5$, calcd 324.1811] $[M-H]^-$, 649.3678 [$C_{34}H_{53}N_2O_{10}$, calcd 649.3700] $[2M-H]^-$; EIMS *m/z* (rel int.) 294 $[M-MeO]^+$ (22), 276 $[M-MeO-H_2O]^+$ (5), 251 $[M-MeO-CO-Me]^+$ (2), 71 $[Et-N{=}C{=}O]^+$ (100).

4.5. Phyllostictine B (**2**)

Compound **2**: $[\alpha]_D^{25}$ −99.8 (*c* 0.07); IR ν_{max} 3407, 1705, 1633, 1444 cm^{-1}; UV λ_{max} nm (log ε) 262 (4.13); ^1H and ^{13}C NMR spectra: see Tables 1 and 2; HRESIMS (+) *m/z*: 914 $[3M+Na]^+$; 617.2984 [$C_{30}H_{46}N_2NaO_{10}$, calcd 617.3050] $[2M+Na]^+$, 320.1443 [$C_{15}H_{23}NNaO_5$, calcd 320.1474] $[M+Na]^+$, 298.1628 [$C_{15}H_{24}NO_5$, calcd 298.1655] $[M+H]^+$; HRESIMS (−) *m/z*: 296.1500 [$C_{15}H_{22}NO_5$, calcd 296.1498] $[M-H]^-$; EIMS *m/z* (rel int.) 298 $[MH]^+$ (8), 280 $[MH-H_2O]^+$ (4), 266 $[M-MeO]^+$ (32), 248 $[M-MeO-H_2O]^+$ (13), 223 $[M-MeO-CO-Me]^+$ (5), 71 $[Et-N{=}C{=}O]^+$ (100).

4.6. Phyllostictine C (**3**)

Compound **3**: $[\alpha]_D^{25}$ −45.5 (0.1); IR ν_{max} 3395, 1704, 1634, 1452 cm^{-1}; UV λ_{max} nm (log ε) 262 (3.60); ^1H and ^{13}C NMR spectra: see Tables 1 and 2; HRESIMS (+) *m/z*: 1046 $[3M+Na]^+$; 705 $[2M+Na]^+$, 364.1707 [$C_{17}H_{27}NNaO_6$, calcd 364.1736] $[M+Na]^+$.

4.7. Phyllostictine D (**4**)

Compound **4**: $[\alpha]_D^{25}$ −70.2 (*c* 0.2); IR ν_{max} 3409, 1707, 1634, 1444 cm^{-1}; UV λ_{max} nm (log ε) 262 (3.25); ^1H and ^{13}C NMR spectra: see Tables 1 and 2; HRESIMS (+) *m/z*: 1040 $[3M+Na]^+$; 701 $[2M+Na]^+$, 362.1548 [$C_{17}H_{25}NNaO_6$] [calcd 362.1580, $M+Na]^+$.

4.8. Acetylation of phyllostictine A

Phyllostictine A (**1**, 3.7 mg) was acetylated with pyridine (20 μL) and Ac_2O (40 μL) at room temperature overnight. The reaction was stopped by addition of MeOH and the azeotrope formed by addition of C_6H_6 was evaporated by a N_2 stream. The oily residue was purified by preparative TLC (silica gel, eluent B) to give the 15-*O*-acetyl and the 11,15-*O*,*O*′-diacetyl derivatives of phyllostictine A (**5** and **6**) both as homogeneous compounds (R_f 0.48 and 0.77, 2.4 and 0.5 mg). Derivative **5** had $[\alpha]_D^{25}$ −55.6 (*c* 0.1); IR ν_{max} 3423, 1725, 1708, 1635, 1440, 1370, 1225 cm^{-1}; UV λ_{max} nm (log ε) 256 (3.58); ^1H and ^{13}C NMR spectra differed from those of **1** for the following signals, δ_H: 5.59 (1H, s, H-15), 2.19 (3H, s, MeCO); δ_C: 172.4 (s, Me*C*O), 71.1 (d, C-15), 20.9 (q, *Me*CO); ESIMS (+) *m/z*: 757 $[2M+Na]^+$, 390 $[M+Na]^+$. Derivative **6** had $[\alpha]_D^{25}$ +125 (*c* 0.04); IR ν_{max} 1739, 1732, 1639, 1442, 1368, 1218 cm^{-1}; UV λ_{max} nm (log ε) 262 (3.34); ^1H and ^{13}C NMR spectra differed from those of **1** for the following signals, δ_H: 5.59 (1H, s, H-15), 5.20 (1H, d, *J*=11 Hz, H-11) 2.13 and 1.99 (3H each, s, 2×MeCO); δ_C: 170.1 and 169.9 (s, 2×Me*C*O), 81.6 (d, C-11), 68.1 (d, C-15), 22.1 and 20.8 (q, 2×*Me*CO); ESIMS (+) *m/z*: 841 $[2M+Na]^+$, 432 $[M+Na]^+$.

4.9. (*S*)-α-Methoxy-α-trifluorophenylacetate (MTPA) ester of phyllostictine A (**7**)

(*R*)-(−)-MTPA−Cl (30 μL) was added to phyllostictine A (**1**, 1.0 mg) and dissolved in dry pyridine (50 μL). The mixture was kept at room temperature. After 1 h, the reaction was

complete and MeOH was added. Pyridine was removed by a N_2 stream. The residue was purified by preparative TLC on silica gel (eluent B) yielding **7** as an oil (R_f 0.36, 0.5 mg): $[\alpha]_D^{25}$ −15.5 (*c* 0.01); IR ν_{max} 3410, 1746, 1718, 1628, 1446, 1272, 1241 cm^{-1}; UV λ_{max} nm (log ε) 263 (3.78); ^1H spectrum differed from that of **1** for the following signals, δ 7.61–7.42 (5H, m, Ph), 5.85 (1H, s, H-15), 4.17 (1H, d, *J*=10.3 Hz, H-11), 3.51 (3H, s, MeO), 1.78 (2H, m, H_2-10), 1.56 and 1.38 (1H each, m, 5.04 H_2-11); ESIMS (+) *m/z* 564 $[M+Na]^+$, 308 $[M+H-PhC(OMe)CF_3COO]^+$.

4.10. (*R*)-α-Methoxy-α-trifluorophenylacetate (MTPA) ester of phyllostictine A (*8*)

(*S*)-(+)-MTPA–Cl (30 μL) was added to phyllostictine A (**1**, 1.0 mg) and dissolved in dry pyridine (50 μL). The reaction was carried out under the same conditions used for preparing **7** from **1**. Purification of the crude residue by preparative TLC on silica gel (R_f 0.36, eluent B) yielded **8** as an oil (0.7 mg): $[\alpha]_D^{25}$ −69.0 (*c* 0.1); UV, IR, and EIMS were very similar to those of **7**; ^1H spectrum differed from that of **1** for the following signals, δ 7.62–7.42 (5H, m, Ph), 5.79 (1H, s, H-15), 4.10 (1H, d, *J*=10.2 Hz, H-11), 3.62 (3H, s, MeO), 1.61 (2H, m, H_2-10), 1.32 and 1.25 (1H each, m, 5.04 H_2-11); ESIMS (+) *m/z* 564 $[M+Na]^+$, 308 $[M+H-PhC(OMe)CF_3COO]^+$.

4.11. Phytotoxic activity

Culture filtrates, organic extracts, their chromatographic fractions, and pure phyllostictines A–D were assayed on *C. arvensis* leaves by puncture assay. The pure toxins and the fractions were first dissolved in a small amount of methanol and then diluted to the desired concentration with distilled water (final concentration of methanol: 1%). Droplets (20 μL) of the assay solutions were applied to punctured detached leaves that were then kept in moistened chambers under continuous light. Symptom appearance was observed 3 days after droplet application. Phyllostictines A–D were tested at concentrations of around 6×10^{-3} M.

4.12. Antimicrobial activity

The antifungal activity of phyllostictines A and B was tested up to 100 μg/disk on *G. candidum*, whereas the antibiotic activity was assayed on *Lactobacillus* sp. and *E. coli*, as previously described.[22]

4.13. Zootoxic assay

The zootoxic activity of phyllostictines A and B was tested on larvae of *A. salina* L. (brine shrimp) at concentrations between 10^{-3} and 10^{-4} M, as previously decribed.[22]

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