JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Phyllostoxin and Phyllostin, Bioactive Metabolites Produced by *Phyllosticta cirsii*, a Potential Mycoherbicide for *Cirsium arvense* Biocontrol

ANTONIO EVIDENTE,*,† ALESSIO CIMMINO,† ANNA ANDOLFI,† MAURIZIO VURRO,§ MARIA CHIARA ZONNO,§ AND ANDREA MOTTA#

Dipartimento di Scienze del Suolo, della Pianta, dell'Ambiente e delle Produzioni Animali, Università di Napoli Federico II, Via Università 100, 80055 Portici, Italy; Istituto di Scienze delle Produzioni Alimentari, CNR, Via Amendola 122/O, 70126 Bari, Italy; and Istituto di Chimica Biomolecolare, CNR, Comprensorio Olivetti, Edificio 70, Via Campi Flegrei 34, 80078 Pozzuoli, Italy

Phyllosticta cirsii, a fungal pathogen isolated from diseased *Cirsium arvense* leaves and evaluated as a biocontrol agent of this noxious perennial weed, produces different phytotoxic metabolites with potential herbicidal activity when grown in liquid cultures. Phyllostictines A−D, four novel oxazatricycloalkenones, were recently isolated from this pathogen and chemically and biologically characterized. Further purification of the same organic extract provided two other metabolites, named phyllostoxin (**1**) and phyllostin (**2**), which were characterized by spectroscopic technique (essentially NMR and MS). Phyllostoxin and phyllostin proved to be a new pentasubstituted bicyclo-octatrienyl acetic acid ester and a new pentasubstituted hexahydrobenzodioxine carboxylic acid methyl ester, respectively. When tested on punctured *C. arvense* leaves, phyllostoxin proved to be highly phytotoxic, causing rapid and large necrosis, whereas phyllostin had no phytotoxicity in this bioassay. This is not surprising, considering the noteworthy structural differences between the two compounds, suggesting the presence of active functional groups in phyllostoxin not present in the other metabolite. These results further support the focused approach of finding novel metabolites with herbicidal properties by looking at the culture extracts of weed fungal pathogens.

KEYWORDS: *Cirsium arvense*; *Phyllosticta cirsii*; phytotoxins; bicyclo-octatrienyl acetic acid ester; hexahydrobenzodioxine carboxylic acid methyl ester; phyllostoxin; phyllostin; bioherbicides

INTRODUCTION

Cirsium arvense (L.) Scop. (commonly called Canada thistle) is a persistent perennial weed that grows vigorously, forming dense colonies. It spreads both by roots growing horizontally that give rise to aerial shoots and by seeds, either by wind or as a contaminant in crop seed. Canada thistle is native to southeastern Europe and the eastern Mediterranean area (*1*). It has spread to most temperate parts of the world and is considered an important weed around the world because it infests many habitats such as cultivated fields, roadsides, pastures, and rangeland, railway embankments, and lawns. It infests at least 27 crops in 37 countries and thrives in temperate regions of the northern hemisphere (*1*). The management of this weed is difficult, and combinations of mechanical, cultural, and chemical methods are more effective than any single method used alone (*2*). Although the use of weed pathogens could be a feasible option for controlling these noxious weeds, no biocontrol agents are available at this time.

Recently, *Stagonospora cirsii* was proposed for the biological control of *C. arvense*, and the main phytotoxin it produced, named stagonolide, was isolated and chemically and biologically characterized (*3*).

The fungus *Phyllosticta cirsii* has been evaluated as another possible biocontrol agent of Canada thistle (*4*). Species belonging to the genus *Phyllosticta* are known to produce bioactive metabolites, including non host phytotoxins, for example, phyllosinol, brefeldin, and PM-toxin isolated by cultures of *Phyllosticta* sp. (*5*), *P. maydis* (*6*), and *P. medicaginis* (*7*), respectively.

The interest in bioactive metabolites produced by weed pathogens as sources of novel natural herbicides (*8, 9*) prompted us to investigate the production of toxins by this species of *Phyllosticta*. Recently, four phytotoxins were isolated from the liquid culture filtrates of a strain of this fungus, chemically identified as novel oxazatricycloalkenones, biologically characterized, and named phyllostictines A−D (*10*).

Considering that the purified extracts were still toxic after the removal of the mentioned phyllostictines A−D, they were

* Author to whom correspondence should be addressed (telephone +39 0812539178; fax +39-081-2539186; e-mail evidente@unina.it).
† Università di Napoli Federico II.
§ Istituto di Scienze delle Produzioni Alimentari, CNR.
# Istituto di Chimica Biomolecolare, CNR.

10.1021/jf0731301 CCC: $40.75
Published on Web 01/16/2008

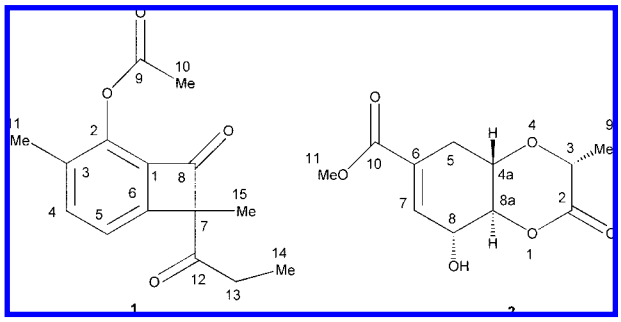

Figure 1. Structures of phyllostoxin and phyllostin (**1** and **2**).

further purified and examined for other compounds. This paper describes the isolation, structural elucidation, and biological characterization of two other new metabolites produced in liquid culture by *P. cirsii* and named phyllostoxin and phyllostin (**1** and **2**, **Figure 1**, respectively). Their structures were determined by extensive use of spectroscopy (essentially NMR and MS techniques).

MATERIALS AND METHODS

Fungus. *P. cirsii*, a fungal pathogen isolated from diseased leaves of *C. arvense,* was supplied by Dr. Alexander Berestetskyi, All-Russian Research Institute of Plant Protection, Pushkin, St. Petersburg, Russia. The strain was maintained in sterile tubes containing potato−sucrose−agar (PDA).

General Experimental Procedure. Melting points of the purified compounds were measured on a Axioskop Zeiss miscroscope (Oberkochen, Germany) coupled with a Metteler FP90 electric hot (Gieben, Germany); optical rotation of each compound was measured in MeOH on a JASCO (Tokyo, Japan) P-1010; IR spectra were recorded as neat on a Perkin-Elmer (Norwalk, CT) Spectrum One FT-IR spectrometer, and UV spectra were taken in MeCN on a Perkin-Elmer Lambda 25 UV−vis spectrophotometer. ^{1}H spectra were recorded at 600 and 400 MHz, in $CDCl_3$, on Bruker (Kalsrhue, Germany) spectrometers. ^{13}C NMR spectra were recorded at 150, 100, and 75 MHz, in the same solvent, using the same instruments. The same solvent was used as internal standard. Carbon multiplicities were determined by DEPT (distortionless enhancement by polarization transfer) experiments (*11*). DEPT, COSY (correlation spectroscopy)-45, TOCSY (total correlation spectroscopy, HSQC (heteronuclear single quantum coherence), HMBC (heteronuclear multiple bond correlation), and NOESY (nuclear Overhauser enhancement spectroscopy) experiments (*11*) were performed using Bruker microprograms. EI and HR EI MS were taken at 70 eV on QP 5050 Shimadzu (Kyoto, Japan) and Fison Prospec (Poole, U.K.) spectrometers, respectively. Electrospray ionization (ESI) MS were recorded on a Perkin-Elmer API 100 LC-MS, using a probed voltage of 5300 V and a declustering potential of 50 V. HR ESI MS spectrum was recorded on Micromass Q-TOF Micro (Milford, MA) instrument. Analytical and preparative TLC were performed on silica gel (Kieselgel 60 F_{254}, 0.25 and 0.50 mm, respectively, Merck, Darmstadt, Germany) or reverse phase (Whatman, KC18 F_{254}, 0.20 mm, Maidstone, U.K.) plates. The spots were visualized by exposure to UV light, I_2 vapor, 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. Column chromatography (CC) was performed on silica gel (Kieselgel 60, 0.063–0.200 mm, Merck). The X-ray analysis of phyllostin was carried out on a colorless crystal obtained from toluene (slow evaporation). Data were acquired on a Nonius Mch3 (Delft, The Netherlands) single-crystal diffratometer (graphite-monochromated Mo Kα radiation). The collection of the data and the assignment of the structure were performed by Prof. A. Tuzi, Dipartimento di Chimica, Università di Napoli Federico II, and will be reported elsewhere.

Production, Extraction, and Purification of Phyllostoxin (1) and Phyllostin (2). For the production of toxins, the fungus was grown as reported previously as well the extraction of the lyophilized phytotoxic fungal culture filtrates (*10*). The organic extract (1.26 g) obtained from the culture filtrates (7.7 L) was purified by silica gel column eluted with $CHCl_3$/i-PrOH (9:1), providing 9 groups of homogeneous fractions. All of the fractions were screened for phytotoxic activity on *C. arvense* leaves as described below. The residue of the second fraction (74.0 mg) was further purified by column chromatography eluted with $CHCl_3$/i-PrOH (9:1) yielding 10 groups of homogeneous fractions. The residues of the sixth (3.6 mg) and seventh (23.2 mg) fractions were combined and purified by preparative TLC on silica gel eluted with $CHCl_3$/i-PrOH (9:1), yielding a homogeneous solid amorphous compound, which was named phyllostoxin (**1**, R_f 0.7, eluent B, 6.0 mg, 0.78 mg/L). The residue of the third fraction of the first column (64.0 mg) was purified by preparative TLC on silica gel eluted with $CHCl_3$/i-PrOH (7:3), yielding a crystalline solid compound, which was named phyllostin (**2**, R_f 0.60, eluent C, 7.0 mg, 0.90 mg/L).

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

C	δ[c]	^{1}H	*J*, Hz	HMBC
1	115.8 (s)			6.15
2	161.0 (s)			2.00, 2.36
3	130.0 (s)			
4	124.0 (d)	6.15 (d)	9.9	2.36
5	138.0 (d)	7.88 (d)	9.9	
6	121.1 (s)			
7	53.6 (s)			6.15, 2.11, 1.95, 1.42, 0.66
8	170.0 (s)			7.88, 2.11, 1.42
9	175.0 (s)			2.00
10	10.6 (q)	2.00 (s)		
11	17.8 (q)	2.36 (s)		
12	201.0 (s)			7.88, 2.11, 1.95, 1.42
13	33.0 (t)	2.11 (dq)	14.8,7.5	1.42, 0.66
		1.95 (dq)	14.8,7.5	
14	9.6 (q)	0.66 (t)	7.5	2.11, 1.95
15	24.0 (q)	1.42 (s)		

[a] 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 protons and the corresponding carbons. [c] Multiplicities determined by DEPT spectrum.

Phyllostoxin (1): amorphous solid; $[\alpha]^{25}_D$ +32.8° (*c* 1.0, $CHCl_3$); IR ν_{max} 1714, 1671, 1658, 1628, 1565 cm^{-1}; UV λ_{max} (log ϵ) nm 321 (3.51), 254 (4.21), 242 (4.21); ^{1}H and ^{13}C NMR spectra: see **Table 1**; EI MS (rel intensity), *m/z* 232 $[M - CO]^+$ (58), 217 $[M - MeCO]^+$ (100), 204 $[M - CH_2{=}C{=}C{=}O]^+$ (66), 189 $[M - CO - MeCO-]^+$ (64), 175 $[M - CO - CH_2{=}C{=}C{=}O]^+$ (30), 161 $[M - MeCO - CH_2{=}C{=}C{=}O]^+$ (23), 43 $[MeCO]^+$ (76); HR ESI MS (+), *m/z* 487.2070 $[C_{28}H_{32}NaO_6$, calcd 487.2097, 2 × M − CO + Na$]^+$, 465.2254 $[C_{28}H_{33}O_6$, calcd 465.2277, 2 × M − CO + H$]^+$, 255 [M − CO + Na$]^+$.

Phyllostin (2): crystalline solid; mp 138–142 °C; $[\alpha]^{25}_D$ −29.4° (*c* 0.1, MeOH); IR ν_{max} 3419, 1733, 1715, 1628, 1303, 1251 cm^{-1} [lit. (*12*), mp 133–135 °C, $[\alpha]_D$ −188.75° (*c* = 2.0 MeOH); IR (nujol) 3410 (OH), 1750 (CO), 1725 (CO); lit. (*13*)., *R* mp 160–165 °C; *S* mp 136–137 °C; lit. (*14*), mp 128–129 °C]; UV λ_{max} nm (log ϵ), 280 (2.97), 242 (sh); ^{1}H and ^{13}C NMR spectra, see **Table 2**; HR EI MS (rel intensity), *m/z* 242.0802 $[C_{11}H_{14}O_6$, calcd 242.0790, M$]^+$ (0.9), 225 $[M - OH]^+$(0.4), 214 $[M - CO]^+$ (62), 211 $[M - MeO]^+$ (0.9), 170 $[M - CO - CO_2]^+$ (44), 142 $[M - 2 \times CO - CO_2]^+$ (66), 95 (100); ESI MS(+), *m/z* 281 $[M + K]^+$, 265 $[M + Na]^+$.

Biological Assay. The phytotoxicity on *C. arvense* host plants, the antimicrobial (including antifungal activity and antibiotic activity against Gram-positive and Gram-negative bacteria), and the zootoxic activity were assayed on *Geotrichum candidum*, *Lactobacillus* sp. and *Escherichia coli* and on brine shrimps (*Artemia salina* L.) as previously reported (*10*).

RESULTS AND DISCUSSION

The liquid culture of *P. cirsii* (7.7 L) was exhaustively extracted as reported under Materials and Methods. The phytotoxic organic extract was purified by a combination of CC and TLC as described under Materials and Methods. Besides phyllostictines A−D, four new oxazatricycloalkenones recently

Table 2. ^{1}H and ^{13}C NMR Data of Phyllostin (**2**)[a,b]

C	δ[c]	^{1}H	*J*, Hz	HMBC
2	169.0 (s)			4.41, 1.58
3	73.1 (d)	4.41 (q)	7.0	3.76, 1.58
4a	70.3 (d)	3.76 (ddd)	9.9, 8, 6, 6.1	4.41, 2.99, 2.40
5	29.8 (t)	2.99 (dd)	17.5, 6.1	6.75, 4.34, 3.76
		2.40 (ddd)	17.5, 9.9, 3.3	
6	132.0 (s)			3.76, 4.54, 2.99, 2.40
7	137.0 (d)	6.75 (br s)	7.4	4.54, 2.99, 2.40
8	70.2 (d)	4.54 (br d)	8.4	4.34
8a	84.3 (d)	4.34 (dd)	8.6, 8.4	6.75, 4.54, 3.76, 2.99
9	17.9 (q)	1.58 (d)	7.0	4.41
10	167.0 (s)			6.75, 3.76, 2.99,
11	52.4 (q)	3.78 (s)		
OH		2.63 (br s)		

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

isolated from the same fungal culture filtrates (*10*), two other compounds were obtained as an amorphous and crystalline solids, respectively, which were named phyllostoxin and phyllostin (**1** and **2**, 0.78 and 0.90 mg/L, **Figure 1**).

Preliminary ^{1}H and ^{13}C NMR investigation showed that the two metabolites were considerably different from phyllostictines A−D.

Phyllostoxin (**1**), the most phytotoxic metabolite together with phyllostictine A, has a molecular formula of $C_{15}H_{16}O_4$ as deduced from HR ESI mass spectrum, consistent with 8 unsaturations four of which were attributed to a tetrasubstituted benzene ring, and the other four to a ketone, an ester and a conjugated carbonyl group in agreement with the typical bands and absorption maxima observed in both IR (*15*) and UV (*16*) spectra.

The ^{1}H spectrum (**Table 1**) showed the doublets (J = 9.9 Hz) of two ortho-coupled aromatic protons at the typical chemical shift values of δ 7.88 and 6.15 (H-5 and H-4) (*17*). Furthermore, the singlets typical of an aromatic and acetyl methyl group were observed at δ 2.36 (Me-11) and 2.00 (Me-10), respectively, together with the triplet (J = 7.5 Hz) of the methyl (Me-14) of a propionyl residue resonating at δ 0.66. In the COSY spectrum (*11*) the latter coupled with the two double quartets (J = 14.8 and 7.5) of the protons of the adjacent methylene group (CH_2-13), which, in turn, resulted also bonded to the saturated ketone group (O═C-12) by the correlations observed in the HMBC spectrum (**Table 1**) (*11*). The couplings observed in the same spectrum allowed assignment the remaining methyl group (Me-15), resonating as singlet at δ 1.42, at the quaternary carbon C-7 (*17*). In the ^{13}C NMR spectrum (**Table 1**) C-7 appeared at the typical chemical shift value of δ 53.6 (*18*) and on the basis of the correlation observed in the HMBC spectrum (**Table 1**), it appeared also bonded to the propionyl residue. C-7 represents the fourth carbon of a disubstituted cyclobutanone ring, which accounted for the remaining unsaturation of **1**. In the HMBC spectrum this latter ketone group (O═C-8), resonating at δ 170.0, coupled with H-5, and with the quaternary methyl group Me-15. The disubstituted cyclobutanone ring joined the benzene ring through its bridgehead quaternary carbons C-1 and C-6, appearing in the ^{13}C NMR spectrum at the typical chemical shifts values of δ 115.8 and 121.1 (*18*). In the same spectrum, the acetyloxy and the aromatic methyl carbons appeared at δ 175.0 (O═C-9), 10.6 (Me-10), and 17.8 (Me-11) and were assigned, on the basis of the coupling observed in the HMBC spectrum, to C-2 and C-3 of the benzene ring, respectively. The latter two carbons were observed at typical chemical shift values of δ 161.0 and 130.0 (C-2 and C-3), and the signals at δ 138.0 and 124.0 were assigned to C-5 and C-4, respectively (*18*), also on the basis of the coupling observed in the HSQC (*11*) spectrum. The signals of the propionyl and tertiary methyl group were observed at δ 201.0 (O═C-12), 33.0 (C-13), 9.6 (C-14), and 24.0 (C-15), respectively, and were attributed also on the basis of the couplings observed in the HSQC spectrum. On the basis of these results phyllostoxin proved to be a new fungal metabolite having the structure of acetic acid 3,7-dimethyl-8-oxo-7-propionyl-bicyclo[4.2.0]octa-1,3,5-trien-2-yl ester (**1**). This structure was confirmed by the results observed in the ESI and EI mass spectra. In fact, the HR ESI MS spectrum, recorded in positive modality, showed sodium clusters formed by the molecular ion after the loss of C═O and those of the corresponding dimer at *m*/*z* 255 and 487.2070, together with the protonated ion of the cited dimer at *m*/*z* 465.2254. Furthermore, the EI MS spectrum did not show the molecular ion, but ions produced by a fragmentation mechanism typical of the functionalities present in **1** (*17*). In fact, the molecular ion by loss of CO produced the ion at *m*/*z* 232 and this, in turn, by alternative loss of MeCO or CH_2═C═C═O residues yielded the ions at *m*/*z* 189 and 175, respectively. The most abundant ion at *m*/*z* 217 was formed from the molecular ion by loss of the acetyl residue and this, in turn, by the loss of CH_2═C═C═O residue generated the ion at *m*/*z* 161. When the molecular ion lost the CH_2═C═C═O residue, the ion at *m*/*z* 204 was obtained. Finally, also the significant acetyl ion was observed at *m*/*z* 43.

Table 3. 2D ^{1}H NOE (NOESY) Data Obtained for Phyllostoxin (**1**) and Phyllostin (**2**)

1		**2**	
considered	effects	considered	effects
7.88 (H-5)	6.15 (H-4)	6.75 (H-7)	4.54 (H-8)
6.15 (H-4)	7.88 (H-5)	4.54 (H-8),	6.75 (H-7), 3.76 (H-4a)
2.36 (Me-11)	2.00 (Me-10)	4.41 (H-3)	3.76 (H-4a), 1.58 (Me-9)
2.11 (H-13)	1.95 (H-13′), 1.42 (Me-15), 0.66 (Me-14)	4.34 (H-8a)	2.40 (H-5′)
1.95 (H-13′)	2.11 (H-13), 1.42 (Me-15), 0.66 (Me-14)	3.78 (Me-11)	2.40 (H-5′)
1.42 (Me-15)	2.11 (H-13), 1.95 (H-13′)	3.76 (H-4a)	4.54 (H-8), 4.41 (H_-3), 2.99 (H-5)
0.66 (Me-14)	2.11 (H-13), 1.95 (H-13′)	2.99 (H-5)	3.76 (H-4a), 2.40 (H-5′)
		2.40 (H-5′)	3.78 (Me-11), 4.34 (H-8a), 2.99 (H-5)

The structure of phyllostoxin appears quite rigid as observed by the inspection of its Dreiding model. The NOE effects observed in the NOESY spectrum (**Table 3**) (*11*) showed the expected proximity of the aromatic protons (H-4 and H-5) and that of the protons of the methylene (CH_2-13) with both the terminal methyl (Me-14) groups of the propionyl residue and the quaternary methyl group (Me-15), as well as that of the aromatic methyl (Me-11) and the methyl (Me-10) of the acetyloxy groups. These results confirmed the structure assigned to **1**.

Phyllostin (**2**) has a molecular formula of $C_{11}H_{14}O_6$ as deduced from HR ESI mass spectrum, consistent with five unsaturations, three of which were attributed to the two ester carbonyl groups and to a trisubstituted conjugated double bond, as also in agreement with the typical bands and absorption maxima observed in both IR (*15*) and UV (*16*) spectra.

The ^{1}H NMR spectrum (**Table 2**) showed the presence of a broad singlet typical of an olefinic proton (H-7) at δ 6.75, which in the COSY spectrum coupled with both the proton (H-8) of a secondary hydroxylated carbon and one proton of the methylene group (CH_2-5), resonating as a broad doublet (J = 8.4) and a doublet of double–doublets (J = 17.5, 9.9, 3.3 Hz) at the expected chemical shift values of δ 4.54 and 2.40, respectively (*17*). The latter (H-5′), in turn, coupled both with the double–doublet (J = 17.5 and 6.1 Hz) of the geminal proton (H-5) at δ 2.99 and with the proton of the adjacent secondary oxygenated carbon (CH-4a), which resonated at δ 3.76 as a doublet of double–doublets (J = 9.9, 8.6, 6.1 Hz), being also coupled with the double–doublet (J = 8.6, 8.4 Hz) at δ 4.34 due to the proton of the adjacent secondary oxygenated carbon (CH-8a). The latter also coupled with the proton H-8 above-described.

These results showed in **2** the presence of a tetrasubstituted cyclohexene ring joined to a trisubstituted 2-oxo-1,4-dioxan ring. In fact, the ^{1}H NMR spectrum also showed the quartet (J = 7.0 Hz) of a secondary oxygenated carbon belonging to this latter ring, which coupled, in the COSY spectrum, with the doublet (J = 7.0 Hz) of the adjacent methyl group (Me-9), and the singlet of an ester methoxy group (Me-11) at δ 3.78 (*17*). The ^{13}C NMR spectrum of **2** (**Table 2**) showed the expected presence of two ester carbonyl groups, the quaternary olefinic carbon, and the methoxy group at δ 169.0, 167.0, 132.0, and 52.4 and were assigned also based on the HMBC correlations (**Table 2**) to C-2, C-10, C-6, and C-11, respectively (*18*). The signals of the secondary olefinic carbon and those of four oxygenated methyne carbons were observed at typical chemical shift values of δ 137.0, 84.3, 73.1, 70.3, and 70.2 and were assigned to C-7, C-8a, C-3, C-4a, and C-8, respectively, on the basis of the HSQC (*11*) couplings. Furthermore, the signals of the methylene and the secondary methyl groups at δ 29.8 and 17.9 were assigned to C-5 and C-9, respectively (*18*). The several interesting correlations observed in the HMBC spectrum (**Table 2**) joined the tetrasubstituted cyclohexane ring to the trisubstituted 2-oxo-1,4-dioxan ring through the bridge-head carbons C-4a and C-8a and located the carboxymethyl and the methyl groups at C-6 and C-3, respectively. On the basis of these results phyllostin is assigned the structure of 8-hydroxy-3-methyl-2-oxo-2,3,4a,5,8,8a-hexahydro-benzo[1,4]dioxine-6-carboxylic acid methyl ester (**2**).

This structure (**2**) was confirmed by the data obtained from the EI and ESI MS spectra. In fact, the HR EI MS spectrum showed the molecular ion at *m*/*z* 242.0802 and ions formed by fragmentation mechanisms typical of the ring nature and functionalitions present in **2** (*17, 19*). The molecular ion losing in succession CO, CO_2, and CO residues produced ions at *m*/*z* 214, 170, and 142, respectively. Alternatively, the molecular ion yielded the ions at *m*/*z* 225 and 211, by loss of OH or MeO residues, respectively. The ESI MS spectrum showed potassium and sodium clusters at *m*/*z* 281 and 265.

On the basis of above NMR data and the several NOE couplings observed in the NOESY spectrum (**Table 3**) the relative stereochemistry of junction between the two rings and the four chiral carbons was assigned as depicted in **2**. As deduced from a Dreiding model inspection, the tetrasubstituted cyclohexene and the trisubstituted 2-oxo-1,4-dioxan rings assume a half-chair and a like-chair conformation, respectively. They appeared to be trans joined, considering the typical axial−axial values (J = 8.6 Hz) measured for the coupling between H-4a and H-8a (*17*). This conformation and the relative stereochemistries of all the chiral centers were definitively assigned by a X-ray diffrattometric analysis of **2** and were found to be 3*R*,4a*S*,8*R*,8a*S* or its enantiomer 3*S*,4a*R*,8*S*,8a*R*. Phyllostin appeared to be the diastereomer of the 5-lactyl shikimate lactone previously isolated from a *Penicillium* sp. (*12*), for which the absolute stereostructure 3*S*,4a*R*,8*R*,8a*R* was established by two independent enantioselective synthesis (*13, 14*). In the same paper Alberg et al. (*13*) also described the preparation of the 3*R*,4a*R*,8*R*,8a*R* diastereomer of **2**. As expected, the spectroscopic (IR, ^{1}H and ^{13}C NMR, and MS) data of phyllostin were similar to those described in literature for the natural (*12–14*) and synthetic (*13*) diastereomers, but the physical (melting point and specific optical rotation) properties appeared to be quite different, as reported under Materials and Methods. Furthermore, the data of the crystalline cells are also quite different with respect to those reported (*20*) of an unidentified diastereomer of **2** previously synthesized by Sprecher and Sprinson (*21*).

When tested on punctured *C. arvense* leaves at a concentration of 10^{-3} M (20 μL/droplet), phyllostoxin proved to be phytotoxic, causing the rapid appearance of large necrosis, similar to those caused by phyllostictine A. On the contrary, phyllostin, assayed at the same concentration, proved to be non-phytotoxic. Neither phyllostoxin nor phyllostin, when assayed at concentrations up to 100 μg/disk, showed antimicrobial activity toward *G. candidum* and Gram-negative and Gram-positive bacteria *Escherichia coli* and *Lactobacillus* sp., respectively. No toxicity was caused by either toxin to brine shrimps (*Artemia salina* L.) larvae when assayed up to 10^{-3} M.

In conclusion, phyllostoxin appeared to be a new phytotoxic bicyclo-octatrienyl derivative lacking antimicrobial or zootoxic activities. Therefore, this toxin could represent a potential new natural herbicide. Further studies are in progress to produce the active compound in larger amounts, allowing a more complete biological characterization. Phyllostoxin appeared to be the first naturally occurring bicyclo-octatrienyl derivative. The other bicyclo-octatrienyl derivatives reported are synthetic or intermediate compounds (*22–24*). Phyllostin, which had no toxicity in any of the assays performed, is one of the possible 16 stereoisomers having the same structure. Another is a fungal metabolite (*12*), whereas the others are all synthetic compounds (*13, 20*).

ACKNOWLEDGMENT

We thank Prof. A. Tuzi, Dipartimento di Chimica, Università di Napoli Federico II, for X-ray analysis and "Servizio di Spettrometria di Massa del CNR" for mass spectra. The assistance of the staff is gratefully acknowledged. The NMR spectra were recorded in the laboratory of the Istituto di Chimica Biomolecolare del CNR, Pozzuoli, Italy. We thank D. Melck, G. Ricciardiello, and C. Iodice for technical assistance.

LITERATURE CITED

(1) Mitich, L. W. Thistles I: *Cirsium* and *Carduus*. *Weed Technol.* **1988**, *2*, 228–229.

(2) Trumble, J. T.; Kok, L. T. Integrated pest management techniques in thistle suppression in pastures of North America. *Weed Res.* **1982**, *22*, 345–359.

(3) Yuzikhin, O.; Mitina, G.; Berestetskiy, A. Herbicidal potential of stagonolide, a new phytotoxic nonenolide from *Stagonospora cirsii*. *J. Agric. Food Chem.* **2007**, *55*, 7707–7711.

(4) Berestetskiy, A.; Gagkaeva, T. Y.; Gannibal, P. B.; Gasich, E. L.; Kungurtseva, O. V.; Mitina, G. V.; Yuzikhin, O. S.; Bilder, I. V.; Levitin, M. M. Evaluation of fungal pathogens for biocontrol of *Cirsium arvense*. In *Proceedings of the 13th European Weed*

Research Society Symposium, Bari, Italy, June 19–23; Bàrberi, P., Bastiaans, L., Christensen, S. Eds.; CCBC: Bari, Italy, 2005; Abstr. 7.

(5) Sakamura, S.; Niki, H.; Obata, Y.; Sakai, R.; Matsumoto, T. Isolation and structure of phytotoxic compounds produced by *Phyllosticta* sp. *Agric. Biol. Chem.* **1969**, *33*, 698–703.

(6) Comstock, J. C.; Martinson, C. A.; Gengenbach, B. G. Host specificity of a toxin from *Phyllostycta maydis* for Texas cytoplasmically male-sterile maize. *Phytopathology* **1973**, *63*, 1357–1361.

(7) Entwistle, I. D.; Howard, C. C.; Johnstone, R. A. W. Isolation of brefeldin A from *Phyllosticta medicaginis*. *Phytochemistry* **1974**, *13*, 173–274.

(8) Evidente, A. In *Natural Products for Pest Management*; Rimando, A. M., Duke, S. O., Eds.; ACS Symposium Series 927; American Chemical Society: Washington, DC, 2006; pp 62–75.

(9) Evidente, A.; Abouzeid, M. A. In *Handbook of Sustainable Weed Management*; Singh, H. P., Batish, D. R., Kohli, R. K., Eds.; Haworth Press: New York, 2006; pp 507–552.

(10) Evidente, A.; Cimmino, A.; Andolfi, A.; Vurro, M.; Zonno, M. C.; Motta, A. Phyllostictines A−D, oxazatricycloalkenones produced by *Phyllosticta cirsii*, potential mycoherbicide of *Cirsium arvense* *Tetrahedron* **2007**, http://dx.doi.org/10.1016/j.tet.2007.12.010

(11) Berger, S.; Braun, S. *200 and More Basic NMR Experiments: a Practical Course*, 1st ed.; Wiley-VCH: Weinheim, Germany, 2004.

(12) Isogai, A.; Washizu, M.; Murakoshi, S.; Suzuki, A. A new shikimate derivative, methyl 5-lactyl shikimate lactone, from *Penicillium* sp. *Agric. Biol. Chem.* **1985**, *49*, 167–169.

(13) Alberg, G. D.; Lauhon, C. T.; Nyfeler, R.; Fässler, A.; Bartlett, P. A. Inhibition of EPSP synthase by analogue of tetrahedral intermediate and EPSP. *J. Am. Chem. Soc.* **1992**, *114*, 335–3546.

(14) Muralidharam, V. B.; Wood, H. B.; Ganem, B. Enantioselective synthesis of (−)-methyl 5-lactylshikimate lactone. *Tetrahedron Lett.* **1990**, *31*, 185–188.

(15) Nakanishi, K.; Solomon, P. H. *Infrared Absorption Spectroscopy*, 2nd ed.; Holden Day: Oakland, CA, 1977; pp 14–53.

(16) Scott, A. *Interpretation of the Ultraviolet Spectra of Natural Products*; Pergamon Press: Oxford, U.K., 1964; pp 45–126.

(17) Pretsch, E.; Bühlmann, P.; Affolter, C. *Structure Determination of Organic Compounds − Tables of Spectral Data*; Springer-Verlag: Berlin, Germany, 2000; pp 161–239, 313−383.

(18) Breitmaier, E.; Voelter, W. *Carbon-13 NMR Spectroscopy*; VCH: Weinheim, Germany, 1987; pp 183–232.

(19) Porter, Q. N. *Mass Spectrometry of Heterocyclic Compounds*, 2nd ed.; Wiley: New York, 1985; pp 335–342.

(20) Chen, C. H.; Low, B. W. Preliminary X-ray crystallographic data for methyl 3-*O*-(1-carboxyethyl) shikimate δ-lactone. *Acta Crystallogr.* **1966**, *20*, 917.

(21) Sprecher, M.; Sprinson, D. B. Private communication, 1962.

(22) Grieco, P. A.; Tahikawa, T.; Schillinger, W. J. Bicyclo[2.2.1]heptanes as intermediates in the synthesis of steroids. Total synthesis of estrone. *J. Org. Chem.* **1980**, *45*, 2247–2251.

(23) Kobayashi, K.; Kanno, Y.; Seko, S.; Suginome, H. Photoinduced molecular transformation. Part 135. New synthesis of taiwanin C and justicin E based on a radical cascade process involving β-scission of alkoxy radicals generated from 3- and 8-aryl-1-ehtyl-1,2-dihydrocyclobuta[b]naphthalen-1-ols prepared by thermolysis of (*Z*)-*tert*-butyl-3-amino-3-(bicyclo[4.2.0]octa-1,3,5-trienyl)propenoates. *J. Chem. Soc. Perkin Trans. 1* **1992**, *22*, 3111–3117.

(24) Kobayashi, K.; Kanno, Y.; Seko, S.; Suginome, H. New general synthesis of *tert*-butyl-3-amino-2-naphthalenecarboxylate by an electrocyclic reaction of *o*-quinonedimethides generated from *tert*-butyl (*Z*)-3-amino-3-(bicyclo[4.2.0]0cta-1,3,5,trienyl-7-yl)prop-2-enoates. *J. Chem. Soc. Chem. Commun.* **1992**, 780–781.

Received for review October 25, 2007. Revised manuscript received December 10, 2007. Accepted December 11, 2007. This work was carried out within the project "Enhancement and Exploitation of Soil Biocontrol Agents for Bio-Constraint Management in Crops" (Contract FOOD-CT-2003-001687), which is financially supported by the European Commission within the 6th FP of RTD, Thematic Priority 5 − Food Quality and Safety. The research was also supported by a grant from Regione Campania L.R. 5/02. Contribution DISSPAPA 159.

JF0731301