Stagonolides B−F, Nonenolides Produced by *Stagonospora cirsii*, a Potential Mycoherbicide of *Cirsium arvense*

Antonio Evidente,*[,†] Alessio Cimmino,[†] Alexander Berestetskiy,[‡] Galina Mitina,[‡] Anna Andolfi,[†] 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, All-Russian Institute of Plant Protection, Russian Academy of Agricultural Science, Pushkin, Saint Petersburg 196608, Russian Federation, and Istituto di Chimica Biomolecolare del CNR, Comprensorio Olivetti, Edificio 70, Via Campi Flegrei 34, 80078 Pozzuoli, Italy

Received June 25, 2007

Stagonospora cirsii, a fungal pathogen isolated from *Cirsium arvense* and proposed as a potential mycoherbicide of this perennial noxious weed, produces phytotoxic metabolites in liquid and solid cultures. Recently, the main metabolite, stagonolide (**1**), with interesting phytotoxic properties, was isolated from a liquid culture and characterized as a new nonenolide. In the present work this same fungus, grown in solid culture, exhibited an increased capacity to produce nonenolides. Five new nonenolides, named stagonolides B−F (**2**–**6**), were isolated and characterized using spectroscopic methods. When tested by a leaf disk puncture assay at a concentration of 1 mg/mL, these compounds showed no toxicity to *C. arvense* and *Sonchus arvensis*, whereas stagonolide (**1**) was highly toxic. Stagonolide (**1**) and stagonolide C (**3**) were weakly toxic to *Colpoda steinii*, a protozoan, when tested at 0.05 mg/mL, with the other stagonolides nontoxic. A number of structure–activity relationship observations were made.

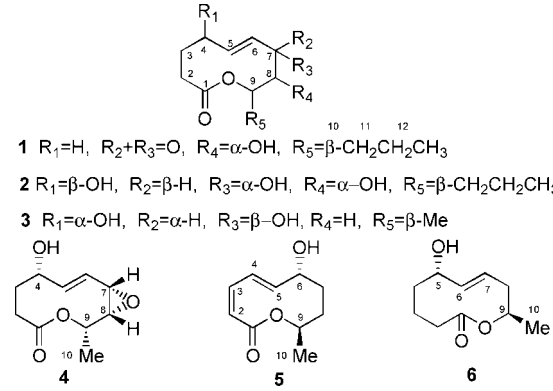
Cirsium arvense (L.) Scop. (Asteraceae) is a perennial and noxious weed found throughout arable land in North America, New Zealand, and Europe. Due to its ability to propagate from lacerated pieces of underground shoots, the plant is resistant to common mechanical weed control methods.[1] Few herbicides are available currently for chemical control of this weed.[2] However, in the future, the phytotoxins of plant pathogens may be able to be used as templates for the development of new selective herbicides.[3–5]

The pycnidial fungus *Stagonospora cirsii* J.J. Davis (anamorphic *Didymella*, *Pleosporales*, Ascomycota) is a foliar pathogen of *C. arvense*, for which its potential for the development of a mycoherbicide has been demonstrated.[6] In a preliminary study, it was found that this fungus is capable of producing phytotoxins because culture filtrates demonstrated phytotoxic activity to the leaves and roots of *C. arvense*.[7] Recently, with the purpose of finding new natural potential herbicides, the main phytotoxic metabolite produced by *S. cirsii* in liquid culture, named stagonolide (**1**), was isolated and characterized as a new nonenolide.[8]

Considering the interesting results obtained and based on previous experience with *Drechslera*,[9] *Phoma*,[10] and *Pyrenophora*[11] species, in which solid culture produced increased and/or different phytotoxins with respect to those isolated from liquid culture, *S. cirsii* was also grown on a solid medium, and the residue obtained by organic solvent extraction was analyzed with the aim of finding new phytotoxic metabolites. This paper describes the isolation, structural elucidation, and biological characterization of five new phytotoxic nonenolides produced in solid culture by *S. cirsii*, named stagonolides B−F (**2**–**6**), respectively. Their structures were determined by extensive use of spectroscopic methods (essentially NMR and MS techniques).

Results and Discussion

A solid culture of *S. cirsii* (1 kg) was extracted exhaustively as described in the Experimental Section. The organic extract, showing a high phytotoxic activity, was purified by a combination of column chromatography and TLC, affording five metabolites, of which the major compound crystallized as a solid, with the remaining compounds obtained as homogeneous amorphous solids. In contrast, stagonolide (**1**), previously isolated as the main phytotoxin from the culture filtrates of the same fungus, was not found.[8] Preliminary ^{1}H and ^{13}C NMR investigations showed that all metabolites have a nonenolide nature, being structurally close to stagonolide, and consequently were named stagonolides B−F (**2**–**6**).



Stagonolide B (**2**) gave a molecular formula of $C_{12}H_{20}O_5$, as deduced from the HRESIMS, consistent with three degrees of unsaturation, of which two are a double bond and a carbonyl lactone group, as deduced from the IR spectrum. Preliminary ^{1}H and ^{13}C NMR investigations showed that the third degree of unsaturation is a nonenolide ring. The IR spectrum also showed bands attributable to hydroxy groups,[12] while the UV spectrum had no absorption maxima above 200 nm. The ^{1}H and ^{13}C NMR spectra showed systems very similar to those of the herbarumins, phytotoxins with potential herbicidal activity isolated from *Phoma herbarum*.[13,14] In particular, stagonolide B (**2**) differed from herbarumin I in the presence of a further secondary hydroxylated carbon located at C-4, as shown by the broad singlet observed at δ 4.63 in the ^{1}H NMR spectrum, which correlated in the COSY spectrum[15] with H-6, H-5, and the protons of CH_2-3 at δ 6.00, 5.65, and 2.10 and 1.88, respectively. This proton (H-4) in the HSQC spectrum[15] coupled with the carbon (C-4) present at the typical chemical shift of δ 68.6.[16] The couplings observed in the COSY and HSQC spectra allowed the assignment of the chemical shifts to all protons and their corresponding carbons (Tables 1 and 2, respectively), and for

* To whom correspondence should be addressed. Tel: +39 081 2539178. Fax: +39 081 2539186. E-mail: evidente@unina.it.
† Università di Napoli Federico II.
‡ Russian Academy of Agricultural Science.
§ Consiglio Nazionale delle Ricerche.

10.1021/np0703038 CCC: $40.75
Published on Web 12/19/2007

Table 1. ^{1}H NMR Data of Stagonolides B−F (**2**–**6**)[a–c]

	2	**3**	**4**	**5**	**6**
position	δ_H *J* (Hz)	δ_H *J* (Hz)	δ_H *J* (Hz)	δ_H *J* (Hz)	δ_H *J* (Hz)
2	2.47 br dd (14.6, 14.3) 2.08 ddd (14.3, 5.4, 2.8)	2.03 (2H) m	2.28 ddd (13.0, 7.4, 1.8) 2.11 dd (13.0, 11.2)	5.84 d (11.6)	2.35 m, 2.00 m
3	2.10 br dd (15.0, 14.6) 1.88 m	2.29 m, 2.03 m	2.05 dd (14.0, 1.8), 2.00 ddd (14.0, 11.2, 4.3)	6.60 br d (11.6)	2.05 (2H)
4	4.63 br s	4.10 m	4.13 ddd (8.4, 7.4, 4.3)	6.12 br d (15.4)	2.00 m 1.60 m
5	5.65 dt (16.1, 2.6)	5.42 dd (15.6, 10.2)	5.52 dd (17.0, 8.4)	5.73 dd (15.4, 9.6)	4.06 ddd (10.2, 9.3, 3.3)
6	6.00 br d (16.1)	5.58 dd (15.6, 9.4)	5.64 dd (17.0, 4.8)	4.24 ddd (9.6, 9.0, 3.8)	5.27 br dd (15.1, 10.2)
7	4.51 br s	4.10 m	3.65 dd (4.8, 3.9)	2.08 ddd (14.2, 9.0, 3.8) 1.73 ddd (14.2, 9.5, 9.0)	5.66 ddd (15.1, 10.7, 3.4)
8	3.58 br dd (9.5, 2.4)	1.88 dd (13.8, 2.6) 1.77 ddd (13.8, 11.2, 2.6)	3.05 dd (3.9, 2.6)	1.85 dd (15.8, 9.0) 1.60 m	2.30 m, 2.05 m
9	4.94 td (9.5, 2.4)	5.14 dq (11.2, 6.2)	5.34 dq (6.7, 2.6)	4.98 dq (11.7, 6.5)	5.15 ddq (12.7, 6.4, 3.4)
10	1.88 m, 1.57 m	1.22 d (6.2)	1.37 d (6.7)	1.21 d (6.5)	1.18 d (6.4)
11	1.37 m, 1.25 m				
12	0.91 t (7.4)				

[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 protons and the corresponding carbons. [c] The assignments are in agreement with values reported for the herbarumins,[13] putaminoxin,[20] and the aspinolides.[23]

Table 2. ^{13}C NMR Data of Stagonolides B−F (**2**–**6**)[a–c]

	2	**3**	**4**	**5**	**6**
position	δ_C mult.[d]	δ_C mult.[d]	δ_C mult.[d]	δ_C mult.[d]	δ_C mult.[d]
1	176.0 qC	174.5 qC	173.5 qC	168.2 qC	174.8 qC
2	27.8 CH_2	34.4 CH_2	31.2 CH_2	125.6 CH	32.1 CH_2
3	31.7 CH_2	31.5 CH_2	35.0 CH_2	139.6 CH	31.5 CH_2
4	68.6 CH	74.4 CH	75.1 CH	126.6 CH	34.3 CH_2
5	127.5 CH	133.0 CH	134.2 CH	140.2 CH	71.8 CH
6	127.1 CH	135.8 CH	128.1 CH	73.7 CH	134.5 CH
7	73.7 CH	72.0 CH	55.4 CH	37.4 CH_2	131.3 CH
8	73.6 CH	43.4 CH_2	58.2 CH	30.4 CH_2	35.0 CH_2
9	70.2 CH	67.7 CH	65.7 CH	73.2 CH	75.4 CH
10	33.6 CH_2	21.3 CH_3	16.2 CH_3	21.4 CH_3	21.7 CH_3
11	18.0 CH_2				
12	12.1 CH_3				

[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 protons and the corresponding carbons. [c] The assignments are in agreement with values reported for the herbarumins,[13]putaminoxin,[20] and the aspinolides.[23] [d] Multiplicities determined by the DEPT spectrum.

stagonolide B (**2**) the structure 4,7,8-trihydroxy-9-*n*-propyl-5-nonen-9-olide was evident. This structure was supported by several correlations observed in the HMBC spectrum[15] (Table S1, Supporting Information).

The other four nonenolides C−F (**3**–**6**) showed in common the lack of an *n*-propyl group at C-9, which was substituted in each case by a methyl group. Stagonolide C (**3**) was assigned a molecular formula of $C_{10}H_{16}O_4$ as deduced from the HRESIMS, consistent with the same three unsaturations of **2**, as deduced from the IR spectrum and preliminary ^{1}H and ^{13}C NMR spectroscopic investigations. The IR spectrum showed also bands attributable to a hydroxy group,[12] while the UV spectrum had no absorption maxima above 200 nm. Inspection of both the ^{1}H and ^{13}C NMR spectra showed that **3** in comparison to **2**, besides the substitution of the *n*-propyl at C-9 with a methyl group, differed in the deoxygenation at C-8. In the ^{1}H NMR spectrum, H-7 appeared as a multiplet overlapped with H-4 at δ 4.10 and in the COSY spectrum coupled with the protons of CH_2-8, resonating as a double-doublet (J = 13.8 and 2.6 Hz) and as a doublet of double-doublets (J = 13.8, 11.2, and 2.6 Hz) at δ 1.88 and 1.77, respectively. The two protons of H_2C-8 and those of Me-10 in the HSQC spectrum coupled with the signals present at the typical chemical shift value of δ 43.4 (C-8) and 21.3, respectively.[16] The couplings observed in the COSY and HSQC spectra allowed the assignment of the chemical shifts to all protons and their corresponding carbons (Tables 1 and 2, respectively), and for stagonolide C the structure 4,7-dihydroxy-9-methyl-5-nonen-9-olide (**3**) was assigned.

Stagonolide D (**4**) gave a molecular formula of $C_{10}H_{14}O_4$, as deduced from HRESIMS, consistent with four unsaturations, with three the same of **2**. The IR spectrum showed bands attributable to a hydroxy group.[12] Both the ^{1}H and ^{13}C NMR spectra showed that **4** in comparison to **2** showed, besides the substitution of the *n*-propyl at C-9 with a methyl group as in **3**, the presence of an epoxy group located between C-7 and C-8. The ^{1}H NMR spectrum (Table 1) showed the presence of two double-doublets (J = 4.8 and 3.9 Hz and J = 3.9 and 2.6 Hz) assignable to H-7 and H-8 at δ 3.65 and 3.05, respectively, which are typical chemical shift values for a 1,2-disubstituted oxirane ring.[17,18] In the COSY spectrum, H-7 coupled with the double-doublet (J = 17.0, 4.8 Hz) of the adjacent olefinic proton (H-6) at δ 5.64, while H-8 coupled with the double-quartet (J = 6.7 and 2.6 Hz) of H-9 at δ 5.34. The two oxirane protons of H-7 and H-8 in the HSQC spectrum coupled with the signals present at the typical chemical shift values of δ 55.4 and 58.2 (C-7 and C-8).[16] The coupling observed in the COSY and HSQC spectra allowed the assignment of the chemical shifts to all protons and their corresponding carbons (Tables 1 and 2, respectively), and for stagonolide D the structure 7,8-epoxy-4-hydroxy-9-methyl-5-nonen-9-olide (**4**) was confirmed.

Stagonolide E (**5**) gave a molecular formula of $C_{10}H_{14}O_3$, as deduced from its HRESIMS, consistent with four unsaturations, with two of them being a lactone and the nonenolide ring as in **2**. The IR spectrum also showed bands attributable to hydroxy and double bonds,[12] while the UV spectrum had an absorption maximum at 250 nm due probably to the extended conjugation of the carbonyl lactone group with one or two double bonds.[19] This was confirmed by the inspection of both ^{1}H and ^{13}C NMR spectra, which when compared to that of **2**, besides the substitution of the *n*-propyl at C-9 with a methyl group as in **3**, showed the presence of signals for a 1,4-disubstituted dienyl residue located between C-2 and C-5. The ^{1}H NMR spectrum (Table 1) of **5** showed the presence of two broad doublets (J = 11.6 Hz and J = 15.4 Hz), a doublet (J = 15.4 Hz), and a double-doublet (J = 15.4 and 9.6 Hz), assigned to H-3, H-4, H-2, and H-5 at the typical chemical shits of δ 6.60, 6.12, 5.84, and 5.73.[18] In the COSY spectrum, besides the couplings between H-2 with H-3 and H-4 with H-5, a very weak coupling (J = 1.3 Hz) was also observed between H-3 and H-4. Furthermore, H-5 coupled with the proton of an adjacent secondary hydroxylated carbon (HO-CH-6), resonating as a doublet of double-doublets (J = 9.6, 9.0, and 3.8 Hz), at δ 4.24. In the HSQC spectrum, the four protons of the 1,4-dienyl systems and

those of the adjacent hydroxylated secondary carbons coupled with the signals observed at δ 140.2, 139.6, 126.6, and 125.6 (C-5, C-3, C-4, and C-2) and δ 73.7 (C-6).[16] The coupling observed in the COSY and HSQC spectra allowed us to assign the chemical shifts to all protons and their corresponding carbons (Tables 1 and 2, respectively), and for stagonolide E the structure 6-hydroxy-9-methyl-2,4-nonadien-9-olide (**5**) was assined.

Stagonolide F (**6**) gave a molecular formula of $C_{10}H_{16}O_3$, as deduced from the HRESIMS, consistent with three unsaturations. These, as deduced from IR and UV spectra and preliminary ^{1}H and ^{13}C NMR investigations, are the same as those observed in putaminoxin, the main phytotoxic nonenolide with potential herbicidal activity isolated from *Phoma putaminum* together with other congeners.[20–22] Both the ^{1}H and ^{13}C NMR spectra of **6** in comparison to putaminoxin[20] showed that the two nonenolides differ only in the substitution of the *n*-propyl at C-9 in putaminoxin with a methyl group in **6**. Accordingly stagonolide F could be formulated as 5-hydroxy-9-methyl-6-nonen-9-olide (**6**).

The relative configuration of the epoxy group in stagonolide D (**4**) was assigned by comparison of the $^3J_{H,H}$ spin systems with the data reported for suitable 1,2-disubstituted *cis*- and *trans*-oxiranes.[17,18] The geometrical isomerism of the double bonds of all nonenolides was determined comparing the coupling constants of the olefinic system considered with values reported in the literature.[18] The double bonds between C(5)−C(6) in stagonolides B−D (**2**–**4**) and between C(4)−C(5) and C(6)−C(7) in stagonolides E (**5**) and F (**6**) are *trans*, while the double bond between C(2)−C(3) in **5** is *cis*, considering the typical $J_{5,6}$, $J_{4,5}$, $J_{6,7}$, and $J_{2,3}$ values, respectively.[18]

The relative configuration of the chiral carbons of stagonolides B−F (**2**–**6**) as depicted in the structural formulas was determined by comparison of the $^3J_{H,H}$ spin systems observed with those of herbarumin I and/or putaminoxin, for which the absolute stereochemistry was independently determined.[13,20] For example, in stagonolide B (**2**), H-4 is α ($J_{4,5}$ = 2.6 Hz), while in putaminoxin, H-5 is β ($J_{5,6}$= 9.4 Hz). In turn H-7 is β ($J_{5,7}$ = 2.6 Hz and $J_{7,8}$ = $J_{7,9}$ = 2.4 Hz) as in herbarumin I ($J_{5,7}$ = $J_{7,8}$ = $J_{7,9}$ = 2.5 Hz); H-8 is β and H-9 is α ($J_{7,8}$ = 2.4 Hz and $J_{8,9}$ = 9.5 Hz) as in herbarumin I ($J_{7,8}$ = 2.5 Hz and $J_{8,9}$ = 9.8 Hz).

The relative configuration assigned to stagonolides B−E (**2**–**5**) is in full agreement with the NOE effects observed in the NOESY spectra,[15] of which the main values are reported in Table S2 (Supporting Information), and with the inspection of Dreiding models. Significant NOESY effects were observed: in the spectrum of **2** between H-8 and H-7, and the protons of CH_2-10 and CH_2-11; in the spectrum of **3** between H-7 and H-9; in the spectrum of **4** between H-8 and H-7 and H-9; and in the spectrum of **5** between H-3 and H-2 and H-4.

Stagonolide F (**6**) appears to be a diastereomer of aspinolide A, a fungal metabolite isolated with other nonenolides and polyketides from *Aspergillus ochraceus* and for which no biological activity was reported.[23] These results were confirmed by the similar spectroscopic data observed for **6** and the aspinolides[23] and by the different optical properties such as the specific optical rotation and CD data (see Experimental Section).

When tested by a leaf disk puncture assay at a concentration of 1 mg/mL, stagonolides B−F (**2**–**6**) showed no toxicity to *C. arvense* and *Sonchus arvensis*, whereas stagonolide (**1**) was highly toxic to both plants. Stagonolide and stagonolide C were practically not toxic to *Colpoda steinii* (a protozoan) tested at 0.05 mg/mL (ca. 2×10^{-4} M), causing inhibition of movement in 50–60% of infusorium cells after 3 h exposure. Stagonolides B−F (**2**–**6**) were nontoxic. Data on the toxic activity of stagonolide (**1**) were in agreement with those from a previous study.[8]

The results of Rivero-Cruz et al.[14] indicated that in herbarumin I hydroxylation of the lactone core at C-2 decreased the resultant phytotoxic activity. Possibly, the hydroxylation of the lactone core at C-4 led to the loss of phytotoxic activity when stagonolide B (**2**) was tested in the leaf disk puncture bioassay. Stagonolide C (**3**) demonstrated the same level of zootoxicity as stagonolide (**1**), but it lost phytotoxic activity. The latter observation can be connected with a change of propyl group at C-9 in **1** to a methyl group in **3**. These results confirm that modifications at the C(2)−C(4) moiety of the nonenolide ring induces a decrease or total loss of phytotoxic activity. Furthermore, stagonolides C−F (**3**–**6**) also differ from the phytotoxic herbarumins I−III[14] and the putaminoxins[20–22] in having a methyl group at C-9 instead of an *n*-propyl group, which appears to be an important structural feature for these two groups of phytotoxins. The functionalization and the conformational freedom of the nonenolide ring are important for the activity of putaminoxins as well as the closely related pinolidoxins,[24] which are phytotoxic metabolites isolated from cultures of *Ascochyta pinodes* Jones, a fungal pathogen for pea (*Pisum sativum* L.).[25,26] The previous results support the observed total loss of phytoxicity for stagonolides D (**4**) and E (**5**) compared to stagonolide (**1**). In fact, the two nonenolides **4** and **5**, which besides the substitution of the *n*-propyl residue at C-9 with a methyl group, possessed marked changes of both funtionalization and conformational freedom of the nonenolide ring, when compared with **1**.

Macrolides and, in particular, nonenolides are quite common as naturally occurring compounds. The nonenolides most closely related to compounds **2**–**6** are the putaminoxins and the herbarumins isolated as phytotoxins with potential herbicidal activity from *Phoma putaminum*[20–22] and *P. herbarum*,[13,14] respectively. Other phytotoxins are the pinolidoxins and aspinolides A−C, isolated from *A. pinodes*[25,26] and *A. ochraceus*,[23] respectively. Putaminoxin and pinolidoxin are potent inhibitors of phenylalanineammonio lyase (PAL), which plays an important role as a plant defensive mechanism.[27]

Experimental Section

General Experimental Procedures. Optical rotations were 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 MeOH solution. IR spectra were recorded 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. ^{1}H and ^{13}C NMR spectra were recorded at 600 MHz and at 150 and 75 MHz, respectively, in $CDCl_3$ on Bruker spectrometers. The same solvent was used as internal standard. Carbon multiplicities were determined by DEPT spectra.[15] DEPT, COSY-45, HSQC, HMBC, and NOESY experiments[15] were performed using Bruker microprograms. ESIMS and HRESIMS were recorded on a Micromass Q-TOF Micro instrument. Analytical and preparative TLC were performed on silica gel (Merck, Kieselgel 60 F254, 0.25 and 0.50 mm, respectively) or reversed-phase (Whatman, KC18 F254, 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. Silica gel (Merck, Kieselgel 60, 0.063–0.200 mm) was used for column chromatography.

Fungal Strain. *Stagonospora cirsii* was originally isolated from diseased leaves of *Cirsium arvense* (L.) Scop. and was maintained in the culture collection of the All-Russian Institute of Plant Protection, St. Petersburg, Russia (No. C-163). Before use, the fungus was stored in tubes on potato-sucrose medium at 5 °C.

Extraction and Isolation. The fungus was grown on autoclaved millet in 1000 mL Erlenmeyer flasks (millet 100 g, water 60 mL) for 14 days in the darkness. Fungal metabolites were extracted from dry mycelium according to a protocol of Evidente et al.,[11] which was slightly modified. The dried material (1000 g) was extracted with acetone–water (1:1). After evaporation of acetone, NaCl (300 g/L) was added to the aqueous residue, and the latter was extracted with EtOAc. The organic extracts were combined, dried (Na_2SO_4), and evaporated under reduced pressure, yielding a brown, oily residue.

The organic extract (1 g) obtained from the culture (1 kg) was purified by silica gel column chromatography eluted with $CHCl_3$−*i*-PrOH (9:1), obtaining 13 groups of homogeneous fractions. The residues of the second and third fractions were combined (353 mg) and further purified by column chromatography, eluted with EtOAc−*n*-hexane (65:35), yielding 13 further groups of homogeneous fractions.

The residue (65 mg) of the fourth fraction was purified by preparative TLC on silica gel, eluting with EtOAc−*n*-hexane (7:3), which gave four bands. The second of these (R_f 0.33; 10.7 mg) was further purified by preparative TLC on a reversed-phase plate, using as eluent MeOH−H_2O (1.5:1), to yield a homogeneous oily compound (R_f 0.31; 1.6 mg), stagonolide F (**6**), and another band (8.3 mg), which was further purified under the same conditions, yielding another homogeneous oily compound (R_f 0.43; 2.9 mg), stagonolide E (**5**). The residue (49 mg) of the seventh fraction of the same column was purified further by preparative TLC on silica gel, using as eluent $CHCl_3$−*i*-PrOH (93:7) to give four bands. The major component, stagonolide D (**4**) (R_f 0.39; 18.0 mg), was obtained as a crystalline solid and named stagonolide D (**4**). The residue (39.3 mg) of the 12 fractions of the initial column was purified by preparative TLC on silica gel, using $CHCl_3$−*i*-PrOH (88:12) for elution, to yield six bands. The fifth band (R_f 0.19; 15.2 mg) was further purified by preparative TLC on silica gel, eluted with EtOAc−MeOH (5:5), yielding two homogeneous oily compounds, stagonolides B and C (**2** and **3**, R_f 0.85 and 0.84; 2.0 and 8.0 mg, respectively).

Stagonolide B (2), (5*β*,8α,9α,10*β*)-5,8,9-trihydroxy-10-propyl-3,4,5,8,9,10-hexahydro-oxecin-2-one: colorless oil; $[\alpha]^{25}_D$ +20 (*c* 0.1); UV λ_{max} < 200 nm; IR ν_{max} 3388, 1710, 1595, 1232 cm^{-1}; ^1H and ^{13}C NMR spectra, see Tables 1 and 2; HRESIMS (+) *m/z* 267.1219 $[M + Na]^+$ (calcd for $C_{12}H_{20}NaO_5$, 267.1208).

Stagonolide C (3), (5α,8*β*,10*β*)-5,8-dihydroxy-10-methyl-3,4,5,8,9,10-hexahydro-oxecin-2-one: colorless oil; $[\alpha]^{25}_D$ +48 (*c* 0.2); UV λ_{max} < 200 nm; IR ν_{max} 3358, 1723, 1239 cm^{-1}; ^1H and ^{13}C NMR spectra, see Tables 1 and 2; HRESIMS (+) *m/z* 223.0946 $[M + Na]^+$ (calcd for $C_{10}H_{16}NaO_4$, 223.0935), 239 $[M + K]^+$.

Stagonolide D (4), (1α,2α,7α,10α)-7-hydroxy-2-methyl-3,11-dioaxabicyclo[8.1.0]undec-8-en-4-one: white, crystalline solid; $[\alpha]^{25}_D$ −82 (*c* 0.2); UV λ_{max} < 200 nm; IR ν_{max} 3434, 1732, 1643, 1221 cm^{-1}; ^1H and ^{13}C NMR spectra, see Tables 1 and 2; HRESIMS (+) *m/z* 221.0781 $[M + Na]^+$ (calcd for $C_{10}H_{14}NaO_4$, 221.0790).

Stagonolide E (5), (7α,10*β*)-7-hydroxy-10-methyl-7,8,9,10-tetrahydro-oxecin-2-one): colorless oil; $[\alpha]^{25}_D$ −186 (*c* 0.2); UV $\lambda_{max}(\log \epsilon)$ 250 (3.37) nm; IR ν_{max} 3399, 1718, 1651, 1605, 1254 cm^{-1}; ^1H and ^{13}C NMR spectra, see Tables 1 and 2; HRESIMS (+) *m/z* 205.0852 $[M + Na]^+$ (calcd for $C_{10}H_{14}NaO_3$, 205.0841).

Stagonolide F (6), (6α,10*β*)-6-hydroxy-10-methyl-3,4,5,6,9,10-hexahydro-oxecin-2-one): colorless oil; $[\alpha]^{25}_D$ −27 (*c* 0.1); CD $[\theta]^{25}$ nm 209.6 (+135 165), 254 (+19 474) [lit.[23] for aspinolide: $[\alpha]^{23}_D$ −43.8 (*c* 0.3, MeOH); CD (MeOH) λ_{extr} $[\theta]^{22}$ 209.8 nm (−6800), 283.2 (+740)]; UV λ_{max} < 200 nm; IR ν_{max} 3375, 1729, 1663, 1237 cm^{-1}; ^1H and ^{13}C NMR spectra, see Tables 1 and 2; HRESIMS (+) *m/z* 207.0989 $[M + Na]^+$ (calcd for $C_{10}H_{16}NaO_3$, 207.0997).

Leaf Disk Puncture Bioassay. Disks of 1 cm in diameter were cut out from well-expanded leaves of *Cirsium arvense* and *Sonchus arvensis* grown in a greenhouse. The disks were placed on moistened filter paper in transparent plastic boxes and wounded with a sharp needle in the center. Samples of the test compounds were dissolved in MeOH and brought up to a final concentration of 1 mg/mL with distilled H_2O. The concentration of MeOH was 2% v/v, which is nontoxic to leaves of both weeds in the control. A drop of test solution (10 μL) was placed in the leaf disk center. The treated disks were incubated under alternate artificial light and temperature: 8 h in darkness at 20 °C and 16 h under light at 24 °C. After 48 h of incubation, the leaf disk necrotic area was measured.

Zootoxic Activity. Stagonolides A−F (**1**–**6**) were assayed on the infusorium, *Colpoda steinii*.[28] Standard Lozina-Lozinskogo medium (2 mL) was added to the dried infusorium culture containing about 5000 cells per mL, and then the resulting suspension was incubated for 24 h at 25 °C before use. The toxin solution in 4% EtOH was added to the infusoria suspension (1:1 v/v) to produce a final concentration of 0.05 mg/mL. After incubation (from 3 to 180 min), the number of immobile cells (%) was counted. In the control treatment, *C. steinii* culture was prepared in 4% EtOH. If 100% of the infusoria cells became immobile within a 3 min exposure with the toxin, the substance tested demonstrated strong toxicity; if they lost activity in ≥180 min, the test substance would be evaluated as having medium toxicity.

Acknowledgment. The authors thank the staff of the "Servizio di Spettrometria di Massa del CNR" for the mass spectra and Mr. C. Iodice (Istituto di Chimica Biomolecolare del CNR, Pozzuoli, Italy) and Dr. G. Ricciardiello (Dipartimento di Scienze del Suolo, della Pianta, dell'Ambiente e delle Produzioni Animali, Università di Napoli Federico II, Napoli) for technical assistance. The NMR spectra were recorded in the laboratory of the Istituto di Chimica Biomolecolare del CNR, Pozzuoli, Italy. The advice of Dr. O. Yuzikhin (All-Russian Institute of Plant Protection) is gratefully acknowledged. This work was carried out within the project "Enhancement and Exploitation of Soil Biocontrol Agents for Bio-Constraint Management in Crops" (contract no. FOOD-CT-2003-001687) and supported financially by the European Commission within the 6th FP of RTD, Thematic Priority 5 Food Quality and Safety. The information/opinions provided in the paper do not necessarily represent the official position/opinion of the European Commission. Contribution DISSPAPA 154.

Supporting Information Available: Tables S1 and S2 containing HMBC and NOSESY NMR spectroscopic data for compounds **2**–**5**, respectively. This information is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Mikhailova, N. F.; Tarasov, Á. V. *Botanicheskiy J.* **1989**, *74*, 509–514.
(2) Donald, W. W. *Rev. Weed Sci.* **1990**, *5*, 193–250.
(3) Kenfield, D.; Bunkers, G.; Strobel, G.; Sugawara, F. In *Phytotoxins and Plant Pathogenesis*; Graniti, A., Durbin, R. D., Ballio, A., Eds.; Springer-Verlag: Berlin, 1989; pp 319−335.
(4) Evidente, A.; Abouzeid, M. A. In *Handbook of Sustainable Weed Management*; Singh, H. P., Batish, D. R., Kohli, R. K., Eds.; The Haworth Press Inc.: New York, 2006; pp 507–552.
(5) Evidente, A. In *Natural Products for Pest Management*; Rimando, A. M.; Duke, S. O., Eds.; ACS Symposium Series 927; Washington, DC, 2006; pp 62–75.
(6) 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. *Proceedings of 13th European Weed Research Society Symposium*, Bari, Italy, June 19–23, 2005, Abstr. 7.
(7) Mitina, G. V.; Yuzikhin, O. S.; Kozlov, I. D.; Berestetskiy, A. *Proceedings of 13th European Weed Research Society Symposium*; Bari, Italy, June 19–23, 2005, Abstr. 217.
(8) Yuzikhin, O.; Mitina, G.; Beretstetskiy, A. *J. Agric. Food Chem.* **2007**, *55*, 7707–7711.
(9) Evidente, A.; Andolfi, A.; Cimmino, A.; Vurro, M.; Fracchiolla, M.; Charudattan, R.; Motta, A. *Phytochemistry* **2006**, *67*, 2281–2287.
(10) Evidente, A.; Andolfi, A.; Vurro, M.; Zonno, M. C.; Motta, A. *J. Nat. Prod.* **2003**, *66*, 1540–1544.
(11) Evidente, A.; Andolfi, A.; Vurro, M.; Zonno, M. C.; Motta, A. *Phytochemistry* **2002**, *60*, 45–53.
(12) Nakanishi, K.; Solomon, P. H. *Infrared Absorption Spectroscopy*, 2nd ed.; Holden Day: Oakland, CA, 1977; pp 17–44.
(13) Rivero-Cruz, J. F.; García-Aguirre, G.; Cerda-García-Rojas, C. M.; Mata, R. *Tetrahedron* **2000**, *56*, 5337–5344.
(14) Rivero-Cruz, J. F.; Macías, M.; Cerda-García-Rojas, C. M.; Mata, R. *J. Nat. Prod.* **2003**, *66*, 511–514.
(15) Berger, S.; Braun, S. *200 and More Basic NMR Experiments: a Practical Course*, 1st ed.; Wiley-VCH: Weinheim, 2004.
(16) Breitmaier, E.; Voelter, W. *Carbon-13 NMR Spectroscopy*; VCH: Weinheim, 1987; pp 183–280.
(17) Batterham, T. J. *NMR Spectra of Simple Heterocycles*; John Wiley & Sons: New York, 1972; pp 365–419.
(18) Pretsch, E.; Bühlmann, P.; Affolter, C. *Structure Determination of Organic Compounds−Tables of Spectral Data*; Springer-Verlag: Berlin, 2000; pp 161–243.
(19) Scott, A. I. *Interpretation of the Ultraviolet Spectra of Natural Products*; Pergamon Press Ltd.: Oxford, UK, 1964; pp 45–88.
(20) Evidente, A.; Lanzetta, R.; Capasso, R.; Andolfi, A.; Bottalico, A.; Vurro, M.; Zonno, M. C. *Phytochemistry* **1995**, *40*, 1637–1641.
(21) Evidente, A.; Lanzetta, R.; Capasso, R.; Andolfi, A.; Bottalico, A.; Vurro, M.; Zonno, M. C. *Phytochemistry* **1997**, *44*, 1041–1045.
(22) Evidente, A.; Capasso, R.; Andolfi, A.; Vurro, M.; Zonno, M. C. *Phytochemistry* **1998**, *48*, 941–945.
(23) Fusher, J.; Zeeck, A. *Liebigs Ann. Rec.* **1997**, 87–95.
(24) Evidente, A.; Lanzetta, R.; Capasso, R.; Vurro, M.; Bottalico, A. *Phytochemistry* **1993**, *34*, 999–1003.
(25) Evidente, A.; Capasso, R.; Andolfi, A.; Vurro, M.; Zonno, M. C. *Nat. Toxins* **1998**, *6*, 183–188.
(26) Evidente, A.; Capasso, R.; Abouzeid, M. A.; Lanzetta, R.; Vurro, M.; Bottalico, A. *J. Nat. Prod.* **1993**, *56*, 1937–1943.
(27) Vurro, M.; Ellis, B. E. *Plant Sci.* **1997**, *126*, 29–38.
(28) GOST 13496.7-97. In *Methods for the Determination of Toxicity*; Standards Publishing House: Moscow, Russia, 1997.

NP0703038