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Pinolide, a New Nonenolide Produced by *Didymella pinodes*, the Causal Agent of Ascochyta Blight on *Pisum sativum*

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Supporting Information

ABSTRACT: An aggressive isolate of *Didymella pinodes* isolated from pea (*Pisum sativum*) produced four different metabolites in vitro. The metabolites isolated from the culture filtrates were characterized by spectroscopic and optical methods. A new nonenolide, named pinolide, was isolated and characterized as (2*S**,7*R**,8*S**,5*E*,9*R**)-2,7,8-trihydroxy-9-propyl-5-nonen-9-olide. Pinolidoxin, the main toxin produced by *D. pinodes*, was also isolated together with two other closely related nonenolides, identified as herbarumin II and 2-epi-herbarumin II. Herbarumin II and 2-epi-herbarumin II have been previously isolated from the fungi *Phoma herbarum* and *Paraphaeosphaeria recurvifoliae*, respectively, but described here to be isolated for the first time from *D. pinodes*. When tested on leaves of the host plant and other legumes and weeds, pinolidoxin was phytotoxic in all of the plant species, whereas the other three nonenolides did not produce any symptoms. The importance of the stereochemistry of the hydroxy group at C-7 on phytotoxicity also is discussed.

KEYWORDS: *Didymella pinodes, Pisum sativum, phytotoxins, nonenolides, pinolide*

INTRODUCTION

Previous work on the solid culture of fungi *Didymella pinodes* (telemorphic stage of *Ascochyta pinodes*) and *Ascochyta pisi*, both responsible for the ascochyta blight of pea (*Pisum sativum*), led to the isolation of several phytotoxins. First from *D. pinodes* and *A. pisi* the main phytotoxins pinolidoxin (**1**, Figure 1) and ascosalitoxin (**2**, Figure 1) were characterized as a new nonenolide, namely, 2-(2,4-hexadienoyloxy)-7,8-dihydroxy-9-propyl-5-nonen-9-olide,[1] and a new trisubstituted derivative of salicylaldehyde, namely, 2,4-dihydroxy-3-methyl-6-(1,3-dimethyl-2-oxopentyl)benzaldehyde,[2] respectively. Furthermore, investigation of the same organic extract of *D. pinodes* allowed for the isolation of three minor and new pinolidoxins, which were characterized as the 7-epi-, 5,6-dihydro-, and 5,6-epoxy-pinolidoxin (**3**−**5**, Figure 1). When assayed on pea and bean leaves the 7-epi- and the 5,6-dihydro-pinolidoxin caused necrotic lesions, although to a lesser extent than pinolidoxin, whereas 5,6-epoxypinolidon was inactive.[3]

Subsequently, putaminoxin (**6**, Figure 1), another new nonenolide, was isolated as the main phytotoxin from *Phoma putaminum*, a fungus proposed as a potential mycoherbicide for the biocontrol of the noxious pasture weed annual fleabane (*Erigeron annuus*).[4] By comparison of putaminoxin (**6**) and pinolidoxin (**1**) with other different fungal toxins, both appeared to be potent inhibitors of phenylalanine ammonia-lyase (PAL) activity, an enzyme that plays a key role in the phenylpropanoid defense metabolism of higher plants. These results indicate that pinolidoxin, as well as putaminoxin, are potent inhibitors of induced PAL activity without displaying any effect on the growth and viability of the cells.[5] The prospect of developing compounds that interfere with plant self-defense mechanism renders this class of compounds highly promising as lead structures in the search for novel herbicidal agents.

Considering this potential practical application in agriculture, some attempts were made for the partial[6] and total stereoselective synthesis[7,8] of pinolidoxin (**1**) as well as herbarumin I and II, and its 2-epimer (**7**−**9**, Figure 1), which are closely related phytotoxic nonenolides with potential herbicide activity that were isolated from *Phoma herbarum*[9] and *Paraphaeosphaeria recurvifoliae*,[10] respectively. Fürstner et al.[7] not only achieved the total synthesis of pinolidoxin (**1**) and herbarumin I and II and its 2-epimer (**7**−**9**) but also revised their interpretation of the ^{1}H and ^{13}C NMR data. Furthermore, on the basis of dynamic calculations and X-ray diffractometric analyses, they unambiguously assigned to pinolidoxin (**1**) the absolute configuration, which is closely related to the biological activity of the naturally occurring compounds[11] and confirmed the previously assigned structures of herbarumin I (**7**) and II (**8**)[9] and its 2-epimer (**9**).[10] The promising herbicidal properties of the phytotoxic fungal nonenolides and the serious constraint exerted by *D. pinodes* on pea production worldwide[12] have prompted us to further investigate the phytotoxins produced by this fungus.

This investigation characterized a new nonenolide, named pinolide (**10**, Figure 1), isolated together with pinolidoxin (**1**),

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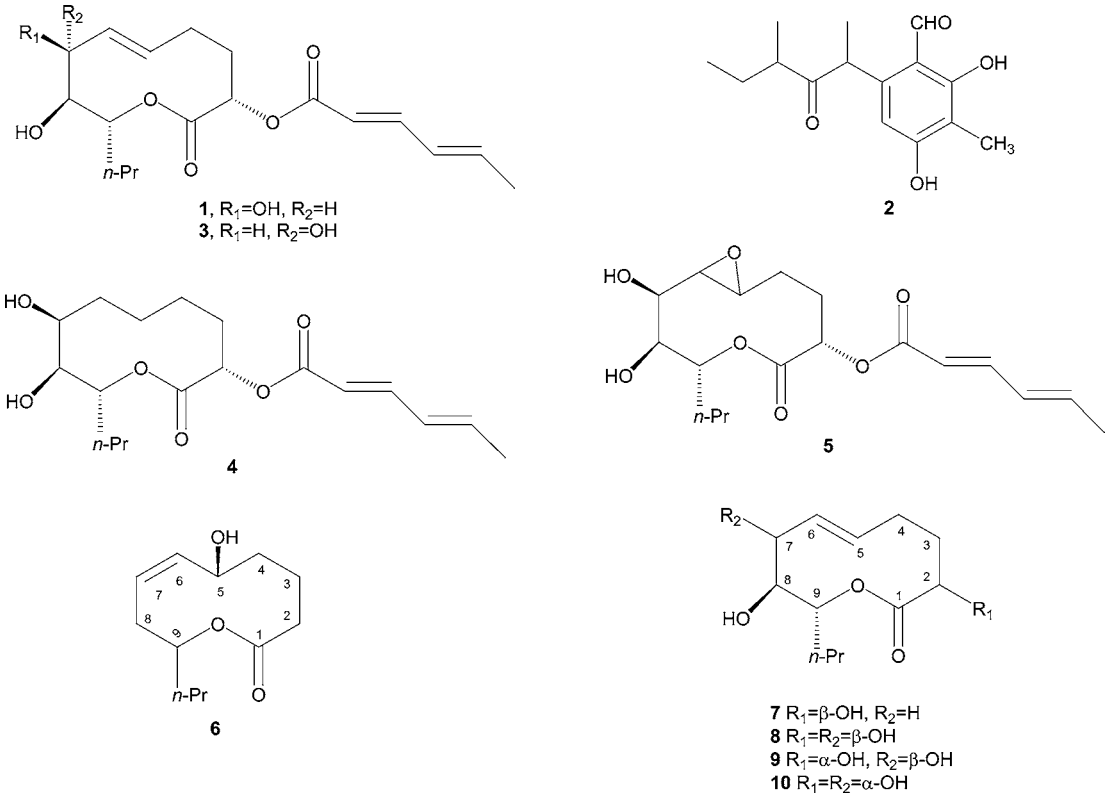

Figure 1. Structures of pinolidoxin (**1**), ascosalitoxin (**2**), 7-epi-, 5,6-dihydro-, and 5,6-epoxy-pinolidoxin (**3**–**5**), putaminoxin (**6**), and herbarumin I, herbarumin II, 2-epi-herbarumin II, and pinolide (**7**–**10**), respectively.

the main phytotoxin, herbarumin II (**8**), and 2-epi-herbarumin II (**9**) from the liquid culture of isolate CO-99 of *D. pinodes*. In addition, to evaluate their specificity and usefulness as herbicides, the effects of these metabolites were assayed on several legumes and weeds.

MATERIALS AND METHODS

General Experimental Procedures. Optical rotation was measured in MeOH, unless otherwise noted, on a Jasco (Tokyo, Japan) polarimeter, whereas the CD spectrum was recorded on a JASCO J-815 CD in MeOH; ^{1}H and ^{13}C NMR spectra were recorded at 400 or 100 MHz, respectively, in $CDCl_3$, on Bruker spectrometers (Kalsruhe, Germany). The same solvent was used as internal standard. Carbon multiplicities were determined by distortionless enhancement by polarization transfer (DEPT) spectra.[13] DEPT, correlation spectroscopy (COSY)-45, heteronuclear single-quantum coherence (HSQC), heteronuclear multiple-bond correlation (HMBC), and nuclear Overhauser enhancement spectroscopy (NOESY) experiments[13] were performed using standard Bruker microprograms. HR and ESIMS spectra were recorded on Waters Micromass Q-TOF Micro and Agilent Technologies 6120 quadrupole LC-MS instruments, respectively. Analytical and preparative thin layer chromatographies (TLC) were performed on silica gel (Kieselgel 60, F_{254}, 0.25 and 0.5 mm, respectively) (Merck, Darmstadt, Germany) plates. The spots were visualized by exposure to UV light at 253 nm or by spraying first with 10% H_2SO_4 in MeOH and then with 5% phosphomolybdic acid in EtOH, followed by heating at 110 °C for 10 min. Column chromatography was performed on a Kieselgel 60, 0.063–0.200 mm silica gel column (Merck).

Fungal Strain, Culture Medium, and Growth Conditions. A monoconidial isolate of *D. pinodes* was obtained from infected pea plants collected in a commercial field at Córdoba, Spain, and deposited in the Collection of the Institute for Sustainable Agriculture, CSIC, Córdoba, Spain (CO-99). The isolate was grown on V8 medium (200 mL V8 juice/L, 30 g agar/L), at 20 °C under a cycle of 12 h light/dark photoperiod. A spore suspension was prepared by flooding the surface of 14-day-old cultures with sterile water, scraping the colony with a needle, and filtering the suspension through two layers of sterile cheesecloth. The concentration of spores in the solution obtained was further determined with a hemocytometer. This suspension was added to a modified Czapek–Dox medium (5% glucose, 0.1% yeast extract, 0.05% K_2HPO_4, 0.2% $NaNO_3$, 0.05% $MgSO_4{\cdot}7H_2O$, and 0.001% $FeSO_4{\cdot}7H_2O$) so that 1×10^6 spores were added per 100 mL of modified Czapek–Dox medium. This liquid culture was incubated for 21 days at 20 °C in the dark on an orbital shaker set at 150 rpm. Then the contents of the flasks were centrifuged at 8000*g*, and the supernatant was filtered through a mesh of 1.0 μm and lyophilized.

Extraction and Purification of *D. pinodes* Metabolites. The lyophilized culture filtrates (2.4 L) were dissolved in $^{1}/_{10}$ of the initial volume with distilled water and extracted with EtOAc (3 × 250 mL). The organic extracts were combined, dried, and evaporated under reduced pressure to yield a brown solid residue (453.0 mg). The residue was tested at a concentration of 5 mg/mL as described below and was found to produce necrotic lesions on *P. sativum*. This residue was then submitted to bioassay-guided fractionation through column chromatography (750 mm × 30 mm) on silica gel, eluted with the $CHCl_3$/*i*-PrOH (9:1, v/v). One hundred and fifty tubes (10 mL each) were checked on TLC and combined. Ten homogeneous fraction groups were collected and screened for their phytotoxic activity. The residue (15.3 mg) of the seventh fraction was further purified by TLC on silica gel, eluted with $CHCl_3$/*i*-PrOH (88:12, v/v), producing two main compounds, both as homogeneous amorphous solids. The more polar compound [R_f 0.27, eluent $CHCl_3$/*i*-PrOH (88:12, v/v), 5.9 mg, 2.5 mg/L] was named pinolide (**10**, Figure 1), whereas the less polar compound [R_f 0.31, eluent $CHCl_3$/*i*-PrOH (88:12, v/v), 5.0 mg, 2.1 mg/L] was identified as 2-epi-herbarumin II (**9**, Figure 1). The residue (37.6 mg) of the sixth fraction of the original column, was further purified by TLC on silica gel using $CHCl_3$/*i*-PrOH (9:1, v/v) as eluent to afford a homogeneous amourphous solid [R_f 0.30, eluent $CHCl_3$/*i*-PrOH (9:1, v/v), 6.2 mg, 2.6 mg/L], identified as herbarumin II (**8**, Figure 1). The residue (122.7.0 mg) of the third fraction of the

original column, containing the main metabolite, was further purified by TLC on silica gel and eluted with *n*-hexane/acetone (6:4, v/v) to yield a homogeneous oily compound (R_f 0.32, 63.0 mg, 26.2 mg/L), which crystallized two times as white needles from *n*-hexane at −20 °C and was identified as pinolidoxin (**1**, Figure 1).

*Pinolide (**10**):* $[\alpha]^{25}_D$ −8.8 (*c* 0.2); UV λ_{max} nm 244 (shoulder); IR 3374, 1710, 1672, 1262 cm^{-1}; ^1H and ^{13}C NMR spectra, see Table 1; HRESI MS (+) spectrum *m/z* 267.1219 [$C_{12}H_{20}NaO_5$, calcd 267.1208, M + Na]$^+$, 245.1399 [$C_{12}H_{21}O_5$, calcd 245.1389, M + H]$^+$, 227 [M + H − H_2O]$^+$ (see also the Supporting Information).

Table 1. ^1H and ^{13}C NMR Data of Pinolide (10)[a,b]

position	δC[b]	δH	*J* (Hz)	HMBC
1	173.0 s[c]			H-9, H-2, H_2-3
2	70.0 d	4.37 d	(5.2, 2.3)	H_2-3
3	31.5 t	2.03 m (2H)		H-2
4	26.7 t	2.43 m		H-5, H-2
		2.11 br d	(13.8)	
5	132.1 d	5.53 ddd	(15.5, 10.0, 3.2)	H_2-4
6	132.7 d	5.56 dd	(15.5, 8.9)	H-8, H-7
7	77.0 d	3.84 br t	(8.9)	H-9, H-8
8	74.5 d	3.45 dd	(9.3, 8.9)	H-9, H-7
9	74.3 d	5.00 td	(9.3, 2.6)	H-8, H_2-10, H_2-11
10	33.8 t	1.94 m		H-9, H_2-11, Me-12
		1.61 m		
11	17.8 t	1.35 m		H-9, H_2-10, Me-12
12	14.0 q	0.93 t	(7.3)	H_2-10, H_2-11

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

*2-Epi-herbarumin II (**9**):* $[\alpha]^{25}_D$ +14° (*c* 0.37) (lit.[10] $[\alpha]^{25}_D$ +15° (*c* 0.66, MeOH)); ^1H NMR spectra were similar to those previously reported;[10] ESI MS (+) *m/z* 267 [M + Na]$^+$, 245 [M + H]$^+$, 227 [M + H − H_2O]$^+$, 199 [M + H − H_2O − CO]$^+$, 181 [M + H − 2 × H_2O − CO]$^+$.

*Herbarumin II (**8**):* $[\alpha]^{25}_D$ +27° (*c* 0.30); CD (MeOH) Δε (nm) 2.60 × 10^4 (207), −3.20 × 10^4 (222) (lit.[9] $[\alpha]_D$ +30.8° (*c* 1 mg/mL), MeOH; CD (MeOH) Δε (nm) 5.31 × 10^4 (209), −2.37 × 10^4 (222); lit.[10] $[\alpha]^{25}_D$ +27° (*c* 0.45), MeOH); ^1H and ^{13}C NMR spectra were similar to those previously reported;[9] ESI MS (+) *m/z* 267 [M + Na]$^+$, 245 [M + H]$^+$.

*Pinolidoxin (**1**):* $[\alpha]^{25}_D$ +142 (*c* 0.43, $CHCl_3$) (lit.[1] $[\alpha]_D$ +142.9 (*c* 0.31, $CHCl_3$)); ^1H and ^{13}C NMR spectra were similar to those previously reported;[1] ESI MS (+) *m/z* 361 [M + Na]$^+$, 339 [M + H]$^+$, 321 [M + H − H_2O]$^+$, 209 [M − H_2O − C_5H_7COO]$^+$, 181 [M − 2 × H_2O − C_5H_7COO]$^+$.

Phytotoxic Assays. The general procedure for all bioassays was as follows: plants were grown in pots, one plant per pot, containing 250 cm^3 of 1:1 sand/peat mixture in a growth chamber at 20 °C with a 12 h light/dark photoperiod until the plants had about seven leaves. Then, one leaflet was detached from each plant and placed onto a square Petri dish (15 × 15 cm) containing 0.4% water/agar medium, the abaxial surface face up. To facilitate toxin penetration, leaf epidermis was cut (three cuts on an area of 3 mm^2) using a scalpel. Metabolites or fractions were dissolved in 5% MeOH and brought up to a final concentration of 4 × 10^{-3} M or 1 mg/mL with distilled water. A drop of 20 μL of the samples was placed over the cuts, and the Petri dishes were subsequently covered with their lids and kept in a growth chamber, set at 20 °C with 12 h light/dark photoperiod. To assess phytotoxicity, the lesions produced by the toxins were considered ellipses, and their longer and shorter diameters were measured. The area of the lesions was calculated as area = Π × r_1 × r_2, where r_1 and r_2 are the long and short radii of the ellipse, respectively. The area of each lesion was scored daily for 4 days.

Organic extracts from the liquid culture filtrates of *D. pinodes*, the chromatographic fractions, and pure compounds (**1**, **8**−**10**) were first bioassayed twice on *P. sativum*. Six replicates per compound were used, and control leaflets were also included. Cuts were performed in these control leaflets as described before a drop of the control treatments was placed on the cuts. In the case of liquid filtrate assays two control treatment were used.

The first consisted in a filtrate from the modified Czapek−Dox medium without the fungi maintained in the same conditions as the medium with the fungus. The second treatment control consisted in the application to the leaflet of the toxin sphaeropsidin A included as positive control. For the pure compounds assay control treatment consisted in the application of a 20 μL drop of 5% methanol.

Table 2. Area of Lesions (mm^2) Caused by Toxicity of Nonenolides Produced by *D. pinodes* in Different Plant Species

species	pinolidoxin	pinolide	herbarumin II	2-epi-herbarumin II
legume crops[a]				
Lupinus albus	73.6 a	0	0	0
Medicago truncatula	49.2 ab	-	-	-
Pisum sativum	45.5 ab	-	-	-
Lens culinaris	45.5 ab	-	-	-
Lathyrus sativus	41.9 ab	-	-	-
Phaseolus vulgaris	34.0 ab	-	-	-
Pisum fulvum	21.5 ab	-	-	-
Cicer arietinum	9.1 b	-	-	-
Vicia faba	7.2 b	-	-	-
weeds[b]				
Chenopodium album	47.8 a	-	-	-
Cirsium arvense	25.0 b	-	-	-
Sonchus arvensis	23.6 b	-	-	-

[a]Data with different letters per column are significantly different (LSD using Bonferroni correction, $P < 0.05$). [b]Data with different letters per column are significantly different (LSD, $P < 0.05$).

Later, to check their specificity, the pure compounds were also assayed on eight additional legume crop species (Table 2). Two different accessions per legume species were included in the study. In addition, to check the effect of these pure compounds as herbicides, they were also tested on three weed species: Canada thistle (*Cirsium arvense*), lamb's quarters (*Chenopodium album*), and perennial sowthistle (*Sonchus arvensis*). For testing the phytotoxicity of the pure metabolites on the different legumes and on the weeds, three to four replications were employed. Leaflets were arranged for each compound in a complete randomized design. Control leaflets were also included in these experiments. Control treatment consisted in the application of a 20 μL drop of 5% methanol.

Statistical analyses were performed using the Statistix 8.0 statistical package (Analytical Software, Tallahase, FL, USA). Before performing analyses of variance, the normality and equality of variances were checked using Shapiro−Wilk's and Levene's tests, respectively. Comparison of means was performed by least significant difference (LSD) when a low number of means was compared (weeds bioassay). When a high number of means were compared (different legumes assay), Bonferroni correction was applied. Null hypotheses were rejected when $P \leq 0.05$.

RESULTS AND DISCUSSION

The liquid culture filtrates of *D. pinodes* were exhaustively extracted with ethyl acetate, and the organic extract, showing high phytotoxic activity on the host plant, was purified by combined column and thin layer chromatography, affording four metabolites, **1** and **8**−**10** (Figure 1), the three minor ones (**8**−**10**) as amorphous solids, and the other, the main phytotoxin (**1**), as an oil, which crystallized as white needles. The preliminary NMR investigation, using both ^{1}H and ^{13}C NMR spectra, showed that the four metabolites are all closely related and belong to the family of nonenolides, which are very well-known as bioactive fungal metabolites.[14,15]

In particular, the metabolite **10**, having a molecular weight of 244 corresponding to a molecular formula of $C_{12}H_{20}O_5$, as deduced from its HR ESI MS spectrum, showed ^{1}H and ^{13}C NMR spectra very similar to those of other nonenolides belonging to pinolidoxin[1−3] and herbarumin[9,10] groups.[7] In particular, the ^{1}H NMR spectrum showed the signals of two olefinic protons (H-5 and H-6), which appeared as a doublet of double doublets (J = 15.5, 10.0, 3.2 Hz) and a double doublet (J = 15.5, 8.9 Hz) at the typical chemical shift values of δ 5.53 and 5.56.[16] These were coupled in the COSY spectrum,[13] respectively, with the protons of adjacent methylene (H_2C-4), which resonated at δ 2.43 and 2.11 (H_2-4) as a multiplet and a broad doublet (J = 13.8 Hz) and with the proton (H-7) of the adjacent secondary hydroxylated carbon (C-7), appearing at δ 3.84 as a broad triplet (J = 8.9 Hz). H-7, also coupled with the proton (H-8) of the adjacent hydroxylated secondary carbon (C-8), resonated at δ 3.45 as a double doublet (J = 9.3 and 8.9 Hz). H-8, in turn coupled with the proton (H-9) of the secondary oxygenated carbon (C-9), which was involved in the closure of the lactone ring. H-9 appeared as double triplet at δ 5.00 (J = 9.3 and 2.6 Hz), also being coupled with the protons (H_2C-10) of the bonded propyl side chain. The two protons of H_2C-10 resonated as two multiplets at δ 1.94 and 1.61, also being coupled with the protons of the adjacent methylene group H_2C-11, which resonated as a multiplet at δ 1.35. The two protons of H_2C-11, in turn, coupled with the protons of the terminal methyl group, were observed at δ 0.93 as a triplet (J = 7.3 Hz). In the COSY spectrum a long-range coupling was observed between H-7 and the protons of H_2C-4. H-2 resonated at δ 4.37 as a double doublet (J = 5.2, 2.3), being coupled with the protons of H_2C-3, observed as multiplet at δ 2.03.[16] These results suggested that **10** is a 2,7,8-trihydroxy-9-propyl-5-nonen-9-olide.

This structure was confirmed by the investigation of its ^{13}C NMR spectrum (Table 1) in which chemical shifts were also assigned on the basis of the coupling observed in its HSQC spectrum.[13] The lactone carbonyl appeared at the typical chemical shift value of δ 173.0, whereas the olefinic and four oxygenated secondary carbons resonated at δ 132.7, 132.1, 77.0, 74.5, 74.3, and 70.0 (C-6, C-5, C-7, C-8, C-9, and C-2, respectively). The methylene carbons of both the nonenolide ring and the propyl group appeared at δ 31.5 and 26.7 and 33.8 and 17.8 (C-3 and C-4 and C-10 and C-11, respectively), whereas the terminal methyl group resonated at δ 14.0.[17]

The structure assigned to pinolide was also confirmed by the couplings observed in the HMBC spectrum[13] (Table 1); of particularly significance was the coupling of the lactone carbonyl C-1 with H-9, H-2, and H_2-3 and those of C-5 with H_2-4, C-6 with H-7 and H-8, and C-9 with H-8, H_2-10, and H_2-11. The data of the HR ESI MS spectrum, which showed the sodium cluster and the pseudomolecular ion at m/z 267.1219 $[M + Na]^+$ and 245.1399 $[M + H]^+$ supported this structure. Furthermore, the pseudomolecular ion by loss of H_2O produced the ion $[M + H - H_2O]^+$ at m/z 227. When recorded in negative modality, the spectrum showed the pseudomolecular ion $[M - H]^-$ at m/z 243. The comparison of the $^{3}J_{H,H}$ observed in the ^{1}H NMR spectrum of pinolide (**10**) with those of herbarumin II (**8**),[7,9] and its 2-epimer (**9**),[7,10] and pinolidoxin (**1**)[1] and its 7-epimer (**3**)[3] allowed the assignment of the relative configuration of **10**. In fact, the constants measured for the coupling between H-2 with both protons of H_2-3 are the same reported for pinolidoxin (**1**), its 7-epimer (**3**) and 2-epi-herbarumin II (**9**), and this was β-located. The constants measured for the coupling between H-7 with both H-6 and H-8 were the same observed in the 7-epi-pinolidoxin (**3**), but differed from those observed in pinolidoxin (**1**), herbarumin II (**8**), and its 2-epimer (**9**), so that H-7 is β-located; the constants measured for the coupling between H-9 with H-8 and H_2-10 were the same observed in pinolidoxin (**1**) and its 7-epimer (**3**) and herbarumin II (**8**) and its 2-epimer (**9**), so that H-8 and H-9 were both α- and β-located, respectively. Finally, the constant measured for the coupling between H-5 and H-6 allowed the assignment of *E*-configuration to this double bond in all four nonenolides above cited and in **10** the relative configuration 2*S**,7*R**,8*S**,5*E*,9*R**, as also depicted in Figure 1. This configuration was also consistent with the couplings observed in its NOESY spectrum.[13] Particularly significant were the couplings observed between H-7 with H-9. Furthermore, H-8 coupled with H-9 and all the protons of side chain, and H-9 coupled only with the methylene protons of the same residue.

Metabolite **9** (Figure 1) had a molecular weight of 244, as deduced from its ESI MS spectrum. Its ^{1}H NMR spectrum and its specific optical rotation (OR) are similar to those of the 2-epi-herbarumin II, a metabolite displaying weak activity against murine tyrosinase purified from a melanoma. It was previously detected together with herbarumin II from the culture filtrates of *P. recurvifoliae*, which was isolated from leaf lesions of pendulous yucca (*Yucca recurvifolia*) in Korea.[10] The structure assigned to **9** was also confirmed by the data from its ESI MS spectrum, which showed, besides the sodium cluster and the pseudomolecular ions at m/z 267 and 245, respectively, significant fragmentation ions. In fact, the pseudomolecular ion produced the ions at m/z 227 $[M + H - H_2O]^+$, 199 $[M + H - H_2O - CO]^+$, and 181 $[M + H - 2 \times H_2O - CO]^+$, by successive losses of H_2O, CO, and H_2O, respectively. Metabolite **9** represents the alcoholic part of pinolidoxin, which in the latter is esterified at C(2)−OH with (2*E*,4*E*)-2,4-hexadienoic acid.

Metabolite **8** (Figure 1), as deduced from its ESI MS, showed the same molecular weight as **9** and **10**. Its ^{1}H and ^{13}C NMR spectra, for which complete assignment of the chemical shifts was also based on the couplings observed in the COSY, HSQC, and HMBC spectra, were similar to those reported for herbarumin II, a phytotoxic metabolite isolated together with the other toxin, herbarumin I (**7**), from the culture filtrates of *P. herbarum*. Identification of **8** as herbarumin II was supported by the similar values of their optical properties (OR and CD) recorded in the same conditions.[9,10] *P. herbarum* occurs worldwide and is a fungus that has shown in vitro inhibition of the growth of the algae *Chlorella pyrenoidosa* and is pathogenic to wild oats (*Avena fatua*) and dandelion (*Taraxacum officinale*) seedlings. Herbarumin I and II caused

significant inhibition of radicle growth of seedlings of prince's feather (*Amaranthus hypochondriacus*), and the level of the activity observed for herbarumin I clearly revealed its potential as a herbicidal agent.[9] Identification of **8** as herbarumin II also was supported by data from its ESI MS spectrum, which showed the same sodium cluster and the pseudomolecular ions observed in the ESI MS spectrum of **9**.

Finally, metabolite **1** had a molecular weight of 388 as deduced from its ESI MS spectrum, which is the same recorded for pinolidoxin. It was previously isolated together with three other minor closed nonenolides from a solid culture extract of another *D. pinodes* strain.[1,3] The absolute configuration of pinolidoxin was successively assigned on the basis of its total synthesis, dynamic computational calculation, and X-ray diffractometric analysis.[7] Compound **1** was definitely identified as pinolidoxin by the identity of its ^{1}H and ^{13}C NMR spectroscopy data and physical properties of those previously reported.[1] The structure assigned to **1** was confirmed by the data of its ESI MS spectrum, which besides the sodium cluster and the pseudomolecular ion at m/z 361 and 339, respectively, showed significant fragmentation peaks. The pseudomolecular and the molecular ions produced the ions at m/z 321 $[M + H - H_2O]^+$, 209 $[M - H_2O - C_5H_7COO]^+$, and 181 $[M - 2 \times H_2O - C_5H_7COO]^+$, by successive losses of H_2O and the carboxy side chain residue (C_5H_7COO) and H_2O, respectively.

When tested on different legumes, pinolidoxin produced necrotic lesions of different sizes on all species. This toxin was also phytotoxic on the weeds tested (Table 2). Control treatment did not produce any symptoms. These results indicate that pinolidoxin is nonhost specific because it caused similar symptoms on host and nonhost species. Our results also demonstrated the potential usefulness of pinolidoxin as a potent herbicide. In contrast, none of the other three metabolites identified produced any symptom in any of the legumes nor on the weeds in our study. Pinolidoxin analogues (**3**−**5**) previously isolated from solid culture of *D. pinodes* were not produced by the aggressive strain used in this study, whereas isolation of pinolide (**10**) and herbarumins (**8** and **9**) from solid culture of *D. pinodes* strain is reported here for the first time. Herbarumin I and II (**7** and **8**) had previously shown significant inhibition of radicle growth of seedlings of *A. hypochondriacus*,[9] whereas 2-epi-herbarumin II (**9**) was only assayed for anticancer activity[10] and no herbicidal effects were reported.[10] Considering the stereostructural features of all the nonenolides involved in this and previous studies, we can explain the lack of activity of pinolide and both herbarumins on the basis of the stereochemistry of the hydroxy group at C-7. In fact, in pinolide (**10**), as well as in 7-epi-pinolidoxin (**3**),[3] the hydroxy group at C-7 is α-located, and they possess null or weak activity. In pinolidoxin (**1**), herbarumin I and II and 2-epi-herbarumin II (**7**−**9**), the hydroxy group at C-7 is β-located, and they possess a strong activity in the systems studied, whereas 2-epi-herbarumin II was never tested. In contrast, the stereochemistry of the hydroxy group at C-2 seems to be less important. In fact, except for herbarumin II, in which the hydroxy group at C-2 is β-located, in the other nonenolides it is α-located, and this does not affect the phytotoxicity. However, taking into account the stereochemistry of the hydroxy groups at C-2 and C-7, only 2-epi-herbarumin II could be a biosynthetic precusor of pinolidoxin, and the fungus could use it to be able to produce pinolidoxin more efficiently. The 2,4-hexadienoic acid esterification of the hydroxy group at C-2 seems important for the activity of **1**. The role of pinolide and herbarumin II could possibly be to invoke a synergistic effect with pinolidoxin, but this can be demostrated only by assaying them in a mixture. However, the plants and the assays used for herbarumins in the previous study are different, and this could explain the lack of activity we have observed. We cannot exclude the possibility that the new pinolide and herbarumins could have some toxic effects when applied to different weeds species or using different application methods.

The results reported in Table 2 for pinolidoxin are original as previously pinolidoxin was tested only on pea and bean crops, whereas the activity of pinolidoxin against the three weeds used was demonstrated here and compared with those of the other nonenolides tested.

The isolation of further new and known nonenolides from an aggressive strain of *D. pinodes* isolated from infected pea in Spain and grown on liquid culture in addition to pinolidoxin, which was previously isolated when the same fungus isolated in Italy was grown on solid culture, was not unexpected. In fact, nonenolides are very common naturally occurring compounds in plants and microrganisms,[14,15] and several of them are reported as fungal phytotoxins with potential herbicidal activity.[1,3,4,9,10,15,18] For some of them, pinolidoxin, putaminoxin, and stagonolide, some results on the mode of action were also reported.[5,19] On the basis of these results and considering that some phytotoxic nonenolides could be obtained in relatively large amount from fermentation or total chemical synthesis,[7,8] a potential practical use of some of them as natural safe herbicides could be hypothesized.

■ ASSOCIATED CONTENT

Supporting Information

IR, UV, 1D and 2D ^{1}H and ^{13}C NMR, and HR ESI MS spectra of pinolide (**10**). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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