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THREE NEW TOXIC PINOLIDOXINS FROM *ASCOCHYTA PINODES*

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ABSTRACT.—Three new pinolidoxins called epi-, dihydro-, and epoxy-pinolidoxin (**2**, **4**, and **5**, respectively) were isolated from *Ascochyta pinodes* grown on wheat, and their structures were determined using spectroscopic and chemical methods. They were characterized as 7-*epi*-, 5,6-dihydro-, and 5,6-epoxy-pinolidoxin, respectively. Assayed on pea and bean leaves, epipinolidoxin and dihydropinolidoxin caused necrotic lesions, whereas epoxypinolidoxin was inactive. Only epipinolidoxin and epoxypinolidoxin were active using the brine shrimp assay.

Studies on the toxic metabolites produced by *Ascochyta pinodes* Jones, the causal agent of anthracnose of pea (*Pisum sativum* L.) on which it causes severe lesions and necrosis of both leaves and pods, recently led to the isolation of the main phytotoxin, called pinolidoxin [**1**], a new tetrasubstituted nonenolide (1). A new investigation of the organic culture extracts showed the presence of at least three minor metabolites which were all structurally closely related to pinolidoxin.

This paper describes the isolation and the chemical and biological characterization of these three pinolidoxins.

The crude oily residue, obtained by extraction with an organic solvent (CH_2Cl_2) of *As. pinodes* cultured on sterilized wheat kernels, was fractionated, using a combination of cc and two preparative tlc steps (see Experimental), to yield pinolidoxin [**1**] (1), and another three uv-absorbing metabolites with chromatographic behavior very similar to that of **1**.

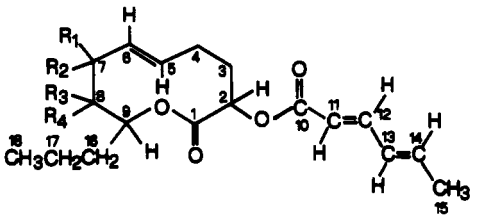
In a preliminary spectroscopic investigation, the three metabolites showed a structure similar to that of pinolidoxin [**1**]. On the basis of their structural relation with **1**, as shown below, they were called epi-, dihydro-, and epoxy-pinolidoxin (**2**, **4**, and **5**, respectively).

Assayed on pea and bean leaves, at 15 μg of toxin/droplet of solution, epipinolidoxin and dihydropinolidoxin caused necrotic lesions similar to those observed for pinolidoxin but broader in size, whereas epoxypinolidoxin was inactive. At a concentration ten times lower, all the metabolites were inactive.

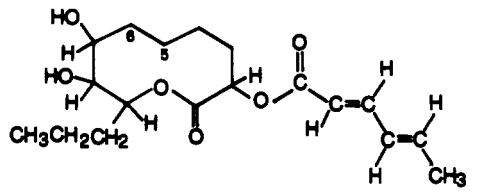
In the brine shrimp assay, epipinolidoxin and epoxypinolidoxin were active at 50 μg/ml, whereas dihydropinolidoxin and pinolidoxin were nearly inactive. At a concentration of 5 μg/ml, all the metabolites were ineffective.

With regard to the antifungal assay, the three new metabolites **2**, **4**, and **5**, as well as pinolidoxin, were inactive at concentrations up to 120 μg/disk.

The three metabolites **2**, **4**, and **5**, as well as **1**, showed ir spectra containing bands characteristic of hydroxyl, conjugated, and saturated ester carbonyl and olefinic groups



1 $R_1=R_3=OH$ $R_2=R_4=H$
2 $R_1=R_4=H$ $R_2=R_3=OH$
3 $R_1=R_4=H$ $R_2+R_3=O-C(Me)_2-O$



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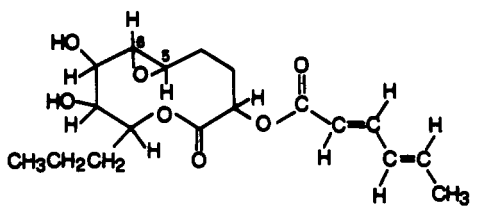

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(1–3). The uv spectrum of epipinolidoxin [**2**] was very similar to that of **1**, while those of **4** and **5** exhibited a decreased absorption at a similar maximum wavelength (1,3,4). The structural relationship of the three metabolites with **1** was deduced from an examination of their ^{1}H- and ^{13}C-nmr spectra.

In fact, the ^{1}H-nmr spectra of **2**, **4**, and **5** (Table 1), as compared to that of **1**, showed the presence of the characteristic signal pattern of both the 2,4-hexadienoyloxy and the propyl residues, the two side chains attached to the macrocyclic ring. These two residues were located on the same carbons (C-2 and C-9, respectively, as in **1**) of the macrolide, as deduced from the multiplicities and the chemical shift values of H-2 and H-9 shown in Table 1.

Moreover, the signal systems of the macrocyclic ring differed in relation to **1**, that of **2** only in the multiplicity of the protons of both the olefinic and the hydroxylated carbons, and those of **4** and **5**, essentially, in the region of the olefinic and the aliphatic protons.

These structural features were also observed by comparing the ^{13}C-nmr spectra of **2**, **4**, and **5** with that of **1** (Table 2).

Epipinolidoxin [**2**], in particular, was structurally closely related to **1**. In fact, its ^{1}H-nmr spectrum (Table 1) differed from that of **1** only in the upfield shift ($\Delta\delta$ 0.61 and 0.13) and the multiplicity of both H-7 and H-8, which appeared as a broad and a sharp triplet centered in **2** at δ 3.83 and 3.39, respectively. In **1**, H-7 and H-8 appeared as a broad singlet and a double doublet, respectively. The different multiplicities of the two systems in **2** were attributable to the different coupling constants measured between H-7 and both H-6 and H-8 ($J_{6,7}=J_{7,8}=8.6$ Hz) compared to the values recorded for the same couplings ($J_{6,7}=1.4$ Hz and $J_{7,8}=2.5$ Hz, respectively) in pinolidoxin [**1**]. These results

TABLE 1. ^{1}H-nmr Data of Pinolidoxin and Epipinolidoxin, Dihydropinolidoxin, and Epoxypinolidoxin (**1**, **2**, **4**, and **5**, respectively).[a]

Proton	Compound			
	1[b]	**2**[c]	**4**[c]	**5**[c]
H-2	5.25 dd (1.7,5.6)	5.26 dd (1.7,5.6)	4.89 t (2.6,2.6)	5.29 dd (1.7,5.6)
H-3	2.20 m	2.20 m	1.95 m	2.90 m
H-3′	2.00 m	2.05 m	1.85 m	2.75 m
H-4	2.41 m	2.40 m	1.55 m	1.85 m
H-4′	2.20 m	2.20 m	1.45 m	1.55 m
H-5	5.53 br t (1.4,15.8,15.8)	5.53 m	1.95 m	3.07 ddd (1.7,2.4,10.8)
H-5′	—	—	1.55 m	—
H-6	5.66 dd (1.4,15.8)	5.66 m	2.30 m	2.86 d (2.4)
H-6′	—	—	2.00 m	—
H-7	4.44 br s (1.4,2.5)	3.83 br t (8.6,8.6)	3.90 br s (2.5)	4.25 d (2.5)
H-8	3.52 dd (2.5,9.4)	3.39 t (8.6,9.4)	3.29 dd (2.5,9.4)	3.71 dd (2.5,9.4)
H-9	5.05 td (2.6,9.4,9.4)	4.92 td (2.6,9.4,9.4)	3.80 td (2.6,9.4,9.4)	5.15 td (2.6,9.4,9.4)
H-11	5.87 d (15.4)	5.86 d (15.4)	5.77 d (15.4)	5.81 d (15.4)
H-12	7.32 br dd (9.8,15.4)	7.31 br dd (9.8,15.4)	7.25 br dd (9.8,15.4)	7.27 br dd (9.8,15.4)
H-13[d]	6.30 m	6.30 m	6.30 m	6.30 m
H-14[d]	6.20 m	6.20 m	6.20 m	6.20 m
Me-15	1.89 br d (5.5)	1.89 br d (5.5)	1.86 br d (5.5)	1.88 br d (5.5)
H-16	1.78 m	1.85 m	1.70 m	1.85 m
H-16′	1.50 m	1.55 m	1.55 m	1.65 m
H-17	1.33 m	1.30 m	1.30 m	1.40 m
H-17′	1.22 m	1.30 m	1.30 m	1.40 m
Me-18	0.87 t (7.3)	0.89 t (7.3)	0.87 t (7.3)	0.88 t (7.3)

[a]The chemical shifts are in δ values (ppm) from TMS and the coupling constants (*J*) are in Hz.
[b]Run at 400 MHz; 2D ^{1}H,^{1}H (COSY) and 2D ^{13}C,^{1}H nmr experiments delineated the correlation among all the protons and the corresponding carbons.
[c]Run at 270 MHz; 2D ^{1}H,^{1}H (COSY) experiments delineated the correlations among all protons.
[d]These attributions may be reversed.

TABLE 2. ^{13}C-nmr Data of Pinolidoxin and Epipinolidoxin, Dihydropinolidoxin and Epoxypinolidoxin (**1**, **2**, **4**, and **5**, respectively).[a]

Carbon	Compound			
	1[b]	**2**	**4**	**5**
C-1	171.9 s	171.2 s	170.1 s	170.9 s
C-2	69.8 d	69.7 d	68.7 d	69.8 d
C-3	29.8 t	29.7 t	29.7 t	28.3 t
C-4	27.4 t	27.6 t	27.6 t	26.5 t
C-5	122.8 d	130.6 d	19.9 t	53.0 d
C-6	132.6 d	133.6 d	25.2 t	61.4 d
C-7	72.9 d	74.5 d	72.1 d	71.0 d
C-8	73.0 d	77.0 d	72.5 d	71.6 d
C-9	71.3 d	73.8 d	71.2 d	70.4 d
C-10	166.1 s	166.0 s	166.1 s	166.0 s
C-11	118.1 d	118.2 d	119.2 d	117.7 d
C-12	145.9 d	145.9 d	144.8 d	146.2 d
C-13[c]	129.7 d	129.7 d	129.7 d	129.7 d
C-14[c]	140.3 d	140.3 d	139.2 d	140.6 d
C-15	18.7 q	18.7 q	18.6 q	17.3 q
C-16	33.6 t	33.4 t	33.6 t	33.7 t
C-17	17.4 t	17.4 t	17.9 t	17.3 t
C-18	13.9 q	13.9 q	14.2 q	14.0 q

[a]The chemical shifts are in δ values (ppm) from TMS. Multiplicities were attributed by DEPT spectra (8).
[b]2D ^{1}H,^{1}H (COSY) and 2D ^{13}C,^{1}H nmr experiments delineated the correlations among all protons and the corresponding carbons.
[c]These attributions may be reversed.

strongly suggested that the stereochemistry of C-7 in **2** was inverted as opposed to that of the same carbon in **1**. Moreover, the coupling constant measured between H-8 and H-9 (J=9.4 Hz) was the same in **1** and **2**; therefore the stereochemistry of C-8 was the same in both metabolites.

These findings suggested for **2** a 7-*epi*-pinolidoxin structure. In fact, the evaluation of coupling constants between H-7 with both H-6 and H-8 (5) combined with an inspection of a Dreiding model of **1** and **2** suggested that H-7 and H-8 should have in **1** an equatorial and an axial configuration and in **2** both should have an axial configuration. From these considerations the 1,2-diol system should have a trans-diequatorial stereochemistry in **2**, which by acid catalyzed reaction with Me_2CO gave rise to a 7,8-isopropylidene derivative **3**, $[M]^+$ *m/z* 378 by eims, similar to the product isolated in the same reaction for **1** in which the 1,2-diol system had a cis stereochemistry (1). This result was not surprising, because other trans-diequatorial 1,2-diols have been transformed in the corresponding isopropylidene derivatives (6,7). The ir spectrum of **3** showed no hydroxy absorptions, while its ^{1}H nmr differed from that of **2** only in the downfield shifts ($\Delta\delta$ 0.15 and 0.20, respectively) of H-5 and H-7, which appeared as a broad and a sharp triplet at δ 5.68 and 4.03, respectively, and in the presence of the two methyl singlets of the isopropylidene group at δ 1.41 and 1.37. Moreover, the H-6 signal appeared as a clear double doublet at δ 5.53. Finally, in **3** the constants measured for the coupling between H-7 with both H-6 and H-8 compared to those observed in the isopropylidene derivative of **1** (1) showed the same difference already observed comparing **2** with **1**.

Therefore, metabolite **2** may be formulated as 7-*epi*-pinolidoxin.

Structure **2** was supported by evidence from the ^{13}C-nmr spectrum (Table 2), which differed with respect to that of **1** in the significant downfield shift ($\Delta\delta$ 7.8) of C-5. This result may be attributed to the epimerization of C-7 in **2**, which determines an equatorial configuration of the attached hydroxyl group. In fact, as observed in cyclohexane derivatives, which seem steric related to the C-5–C-1 moiety of **2**, the introduction of an equatorial hydroxyl group, as at C-7 in **2**, produces a γ shielding significantly less compared with the introduction of an axial OH group as at C-7 in **1** (8).

Further support for the structure of 7-*epi*-pinolidoxin was obtained from its eims, which showed the same molecular ion as observed in **1** at *m/z* 338. The molecular ion, losing H_2O, produced the ion at *m/z* 320, which, in turn, by loss of C_5H_7, C_5H_7CO, and C_5H_7COO residues, yielded the most significant fragmentation peaks at *m/z* 253, 225, and 209, respectively (1,3). In addition, its fabms exhibited the same protonated molecular ion $[MH]^+$ at *m/z* 339 and the same prominent ion *m/z* 321 $[MH-H_2O]^+$ as in **1**.

The ^{1}H-nmr spectrum of dihydropinolidoxin [**4**] (Table 1) showed, compared to that of **1**, the absence of the signal due to the olefinic protons H-5 and H-6, and the presence of other complex systems in the region between δ 2.30 and 1.40. In fact, the 2D ^{1}H,^{1}H-nmr correlation experiment (COSY) (8) led to the assignment of the multiplets at δ 2.30 and 2.00 and at δ 1.95 and 1.55 to the protons of H_2-6 and H_2-5, respectively. Moreover, a significant upfield shift ($\Delta\delta$ 0.86, 0.75, 0.54, and 1.25, respectively) of H-4, H-4′, H-7, and H-9 was observed.

The ^{13}C-nmr spectrum of **4** differed from that of **1** only in the absence of the secondary olefinic carbons attributed in **1** to C-5 and C-6 and in the presence of two other aliphatic methylene groups, resonating at δ 25.2 and 19.9 (C-6 and C-5, respectively).

On the basis of these results, it is possible to suggest the structure of a 5,6-dihydropinolidoxin for metabolite **4**.

This structure was confirmed by the examination of its eims spectrum, which, as expected, showed a molecular ion at *m/z* 340 (*m/z* 338 in **1**) and characteristic peaks due

to fragmentation mechanisms as already observed in **1** (1,3). The most significant peaks were those recorded at *m/z* 322, 279, and 167, generated from the molecular ion by successive losses of H_2O, C_3H_7, and C_5H_7COOH residues, and those at *m/z* 245 and 227 from the parent ion by successive losses of C_5H_7CO and H_2O residues. Moreover, in the fabms, **4** exhibited the protonated molecular ion $[MH]^+$ at *m/z* 341, which, losing in succession H_2O, C_2H_5COOH, and H_2O, yielded the significant peaks at *m/z* 323, 211, and 193, respectively.

Based on these results, dihydropinolidoxin may be formulated as 2-(2,4-hexadienoyloxy)-7,8-dihydroxy-9-propylnonan-9-olide [**4**].

Epoxypinolidoxin [**5**] had a mol wt of 354, as deduced from its eims and fabms, and therefore should differ from **1** by one oxygen atom. An accurate inspection of its ^{1}H-nmr spectrum showed, by comparison to that of **1**, the absence of the signals of the olefinic protons (H-5 and H-6) of the macrocyclic ring and the noteworthy presence of the signals typical of an epoxy group (9,10). In fact, a doublet of double doublets (H-5) and a sharp doublet (H-6) resonated at δ 3.07 and 2.86, respectively. The system is characteristic of a trans-disubstituted oxirane ring, as also deduced from the significant value of the coupling constant ($J_{5,6}$=2.4 Hz) between H-5 and H-6 (9), where the epoxy ring was easily located. This coupling constant is in good agreement with the values recorded for a series of mono- and 1,2-disubstituted oxiranes in which, with the exception of those bearing strong electronegative groups, the J_{cis} (4.0–5.0 Hz) was always more than twice J_{trans} (1.7–2.5 Hz) (5,9,11,12). Moreover, these evaluations were further supported by the fact that a cis stereochemistry, deduced from the vicinal coupling constant (J=4.4 Hz) and confirmed by an X-ray analysis (13), could be assigned to the epoxy group present in the seiricuprolide, a new phytotoxic 14-macrolide recently isolated by one of us from the culture filtrates of *Seiridium cupressi* (14). Finally, the ^{1}H nmr of **5** showed the appearance of a sharp doublet at δ 4.25 assigned to H-7 and the upfield shifts (Δδ 0.56 and 0.65) of the protons of H_2-4.

From these results, the structure of a 5,6-epoxypinolidoxin was deduced for **5**. This result was in full agreement with the typical absorption band observed in its ir spectrum at 1210 cm^{-1} (10) and the signal pattern observed in the ^{13}C-nmr spectrum (Table 2). In fact, epoxypinolidoxin showed, as the only difference from **1**, the absence of the olefinic carbons of the macrolide and the presence of two doublets at δ 61.4 (C-6) and 53.0 (C-5), chemical shift values very typical of carbons in a 1,2-disubstituted oxirane ring (8,10).

Furthermore, the structure assigned to epoxypinolidoxin [**5**] was supported by the evidence obtained from its eims. In fact, the molecular ion present at *m/z* 354, by loss of Me and C_5H_7CHO residues, yielded the ions at *m/z* 339 and 258. By an alternative fragmentation mechanism, the parent ion losing, in succession, the C_5H_7COO residue and H_2O produced the significant ions at *m/z* 243 and 225 (1,3). Finally, the fabms of **5** showed the protonated molecular ion $[MH]^+$ at *m/z* 355.

Therefore, epoxypinolidoxin may be formulated as 2-(2,4-hexadienoyloxy)-5,6-epoxy-9-propylnonan-9-olide [**5**].

In conclusion, this work describes the isolation and the structure determination of three minor toxic metabolites from *A. pinodes*. These, together with the recently reported main phytotoxin called pinolidoxin, represent a new family of 10-macrolides with interesting biological activity.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Optical rotations were measured on a 141 Perkin-Elmer polarimeter in $CHCl_3$ solutions; ir spectra were recorded neat on a Perkin-Elmer FT-IR 1720×spectrometer; uv spectra were measured on a Perkin-Elmer 550 S spectrophotometer in MeCN solutions; ^{1}H- and ^{13}C-

nmr spectra were recorded in $CDCl_3$ at 270 or 400 MHz and 67.92 or 100 MHz, respectively, on Bruker spectrometers, using the same solvent as internal standard. Carbon multiplicities were determined by DEPT spectra (8). DEPT and COSY 45 (correlated spectroscopy) experiments were performed using Bruker microprograms. Eims was measured at 70 eV on a VG TRIO-2000 spectrometer; fabms were recorded on a VG ZAB spectrometer in glycerol/thioglycerol using Xe at 9.5 kV as the bombarding gas. Analytical and preparative tlc were performed on SiO_2 (Merck, Kieselgel 60 F_{254}, 0.25 and 0.50 mm, respectively) or on reversed-phase (Whatman, KC18, F_{254}, 0.20 mm) plates; the spots were visualized by exposure to uv radiation and/or by spraying first with 10% H_2SO_4 in MeOH and then with 5% phosphomolybdic acid in MeOH, followed by heating at 110° for 10 min. Cc was carried out on SiO_2 (Merck, Kieselgel 60, 0.063–0.20 mm).

FUNGUS SPECIES.—*As. pinodes* was isolated from infected pea (*P. sativum*) near Bari, Italy, and deposited in the fungus collection of the Istituto Tossine e Micotossine da Parassiti Vegetali del CNR, Bari, Italy (ITEM 1094). The fungus was cultured on autoclaved wheat.

PRODUCTION, EXTRACTION, AND PURIFICATION OF PINOLIDOXINS.—The growth of *As. pinodes* on sterilized wheat kernels and the extraction procedure of its toxic metabolites were recently described in detail (15). The organic (CH_2Cl_2) extracts (436 mg) obtained from 1 kg of culture were fractionated on a SiO_2 column eluting with $CHCl_3$-iPrOH (19:1) to afford 11 homogeneous fractions. Further purification by preparative tlc [SiO_2, eluent $CHCl_3$-iPrOH (19:1)] of the residue (89.4 mg) left from the sixth fraction yielded pinolidoxin [**1**] (73 mg) as a pure compound. The residues (31.3, 7.7, 15.2, and 19.4 mg, respectively) left from fractions 7–10 of the original column, containing metabolites with R_f values lower (0.31, 0.28 and 0.26, respectively) than that (0.33) of pinolidoxin, were purified by preparative tlc [SiO_2, eluent $CHCl_3$-iPrOH (19:1)]. Four zones, located by exposure to uv light, were scraped off and eluted with the same solvent. Evaporation of the solvent yielded the following residues: a further amount of pinolidoxin [**1**] (R_f 0.33, 20 mg); A (from the upper zone, 8.4 mg), in which the main component had R_f 0.31 by tlc [SiO_2, eluent $CHCl_3$-iPrOH (19:1)]; B (from the intermediate zone, 3.9 mg), containing essentially the metabolite having R_f 0.28 in the above tlc system; C (from the lower zone, 6.4 mg) containing essentially the component with R_f 0.26 by tlc in the above system. Further purification of residues A, B, and C by preparative tlc on reversed-phase plates [H_2O-EtOH (2.3:1)] yielded dihydropinolidoxin **4** (3.9 mg), epipinolidoxin **2** (2.0 mg), and epoxypinolidoxin **5** (4.1 mg), all as homogeneous compounds resisting to crystallization, as ascertained by tlc analysis on SiO_2 [eluent, $CHCl_3$-iPrOH (19:1)] and on reversed-phase [eluent, H_2O-EtOH (2.3:1)] plates.

7-epi-*Pinolidoxin* [**2**].—$[\alpha]^{25}D$ +31.1 (c=0.30); uv λ max nm (log ε) 260 (4.20); ir ν max cm^{-1} 3440, 1722, 1652, 1619, 1260; ^1H nmr see Table 1; ^{13}C nmr see Table 2; eims *m/z* (rel. int.) $[M]^+$ 338 (0.3), $[M-H_2O]^+$ 320 (0.2), $[M-H_2O-C_3H_7]^+$ 253 (0.6), $[M-C_5H_7CO]^+$ 243 (0.3), $[M-H_2O-C_5H_7CO]^+$ 225 (2), $[M-H_2O-C_5H_7COO]^+$ 209 (0.3), $[C_5H_7CO]^+$ 95 (100), $[C_5H_7]^+$ 67 (26); fabms *m/z* (rel. int.) $[MH]^+$ 339 (100), $[MH-H_2O]^+$ 321 (87).

7,8-O,O′-*Isopropylidene*-7-epi-*pinolidoxin* [**3**].—7-*epi*-Pinolidoxin (1.3 mg) in dry Me_2CO (3.5 ml) was stirred with dry $CuSO_4$ (31 mg) under reflux for 2 h. The mixture was filtered and evaporated under reduced pressure to give an oily residue. The latter was purified by preparative tlc (SiO_2, eluent $CHCl_3$), yielding **3** as a pure oil (1.1 mg): uv λ max nm (log ε) 260 (4.12); ir ν max cm^{-1} 1724, 1646, 1619, 1242; ^1H-nmr δ differed from that of **2** in the following signal systems: 5.68 (1H, br t, $J_{4,5}=J_{5,6}$=15.4 Hz, H-5), 5.53 (1H, dd, $J_{5,6}$=15.4 and $J_{6,7}$=8.9 Hz, H-6), 4.03 (1H, t, $J_{6,7}=J_{7,8}$=8.9 Hz, H-7), 3.34 (1H, t, $J_{7,8}$=8.9 and $J_{8,9}$=9.2 Hz, H-8), 1.41 and 1.37 (3H, each, s, O-C(Me)$_2$-O); eims *m/z* (rel. int.) $[M]^+$ 378 (0.6), $[M-Me]^+$ 363 (1.4), $[M-MeCOMe]^+$ 320 (3.6), $[M-H_2O-C_3H_7]^+$ 293 (6), $[M-C_5H_7CO]^+$ 283 (2.7), $[M-H_2O-C_5H_7-MeCOMe]^+$ 235 (6), $[C_5H_7COO]^+$ 111 (15), $[C_5H_7CO]^+$ 95 (100), $[C_5H_7]^+$ 67 (39).

5,6-*Dihydropinolidoxin* [**4**].—$[\alpha]^{25}D$ +32.2 (c=0.17); uv λ max nm (log ε) 255 (3.85); ir ν max cm^{-1} 3404, 1715, 1646, 1623, 1246; ^1H nmr see Table 1; ^{13}C nmr see Table 2; eims *m/z* (rel. int.) $[M]^+$ 340 (0.4), $[M-H_2O]^+$ 322 (0.1), $[M-H_2O-Me]^+$ 307 (0.2), $[M-H_2O-C_3H_7]^+$ 279 (0.4), $[M-C_5H_7CO]^+$ 245 (0.6), $[M-C_5H_7CO-H_2O]^+$ 227 (1), $[M-H_2O-C_5H_7COOH]^+$ 210 (1.5), $[M-H_2O-C_3H_7-C_5H_7COOH]^+$ 167 (4), $[C_5H_7CO]^+$ 95 (100), $[C_5H_7]^+$ 67 (29); fabms *m/z* (rel. int.) $[MH]^+$ 341 (18), $[MH-H_2O]^+$ 323 (100), $[MH-C_5H_7COOH]^+$ 229 (42), $[MH-H_2O-C_5H_7COOH]^+$ 211 (83), $[MH-C_5H_7COOH-2\times H_2O]^+$ 193 (63).

5,6-*Epoxypinolidoxin* [**5**].—$[\alpha]^{25}D$ −5.1 (c=0.24); uv λ max nm (log ε) 259 (3.72); ir ν max cm^{-1} 3445, 1717, 1645, 1619, 1210; ^1H nmr see Table 1; ^{13}C nmr see Table 2; eims *m/z* (rel. int.) $[M]^+$ 354 (4), $[M-Me]^+$ 339 (1.5), $[M-H_2CO-C_3H_7]^+$ 281 (1), $[M-C_5H_7CHO]^+$ 258 (3), $[M-C_5H_7COO]^+$ 243 (2), $[M-C_5H_7COO-H_2O]^+$ 225 (2), $[C_5H_7COO]^+$ 111 (15), $[C_5H_7CO]^+$ 95 (100), $[C_5H_7]^+$ 67 (22); fabms *m/z* (rel. int.) $[MH]^+$ 355 (100).

BIOLOGICAL METHODS.—Each sample was dissolved in a minute amount of MeOH and brought up to the required concentration with H_2O (leaf puncture assay) or sea-water solution (brine shrimp assay).

Leaf puncture assay.—Phytotoxic activity was tested as described by Sugawara *et al.* (16), using a simple leaf puncture assay. A drop of test solution (15 μl) containing 15 μg of toxin was applied on the surface of pea and bean leaves previously punctured with a needle. Solutions containing ten times less toxin were also used. The leaves were then sealed in a moist chamber and incubated for 48 h. The symptoms, appearing as chlorotic spots surrounding the punctures, were revealed after 1 day, and became necrotic later.

Mycotoxic activity.—Mycotoxic activity was tested using the assay on *Artemia salina* (brine shrimp) larvae, according to the method described by Capasso *et al.* (17). Solutions containing 50 and 5 μg of toxin/ml of artificial sea water were used.

Antifungal activity.—Antifungal activity was assayed on *Geotrichum candidum*, as previously described (18). The toxins were assayed up to 120 μg/disk.

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