A New Phytotoxic Nonenolide from *Phoma herbarum*

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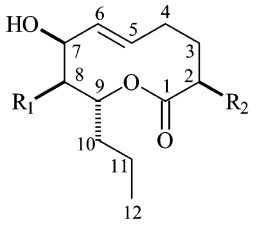
Reinvestigation of the fermentation broth and mycelium of the fungus *Phoma herbarum* led to the isolation of a new phytotoxic nonenolide, namely, (7*R*,9*R*)-7-hydroxy-9-propyl-5-nonen-9-olide, which was designated with the trivial name herbarumin III (**3**). The known compounds herbarumins I (**1**) and II (**2**) were also obtained. The structure of **3** was elucidated by spectroscopic methods and molecular modeling. Compounds **1**−**3** interacted with bovine-brain calmodulin and inhibited the activation of the calmodulin-dependent enzyme cAMP phosphodiesterase.

As part of our search for potential herbicidal agents from Mexican biodiversity, we previously described the isolation and structure elucidation of two novel medium-sized lactones from the mycelium and culture broth of the fungus *Phoma herbarum* Westend (Sphaeropsidaceae).[1] The phytotoxins were characterized as (7*S*,8*S*,9*R*)-7,8-dihydroxy-9-propyl-5-nonen-9-olide (**1**) and (2*R*,7*S*,8*S*,9*R*)-2,7,8-trihydroxy-9-propyl-5-nonen-9-olide (**2**) and given the trivial names herbarumins I and II, respectively. Nonenolides **1** and **2** caused relevant inhibition of radicle growth of seedlings of *Amaranthus hypochondriacus* L. (Amaranthaceae) when tested by the Petri dish bioassay.[1] Both 10-membered-ring lactones were recently synthesized by a concise approach involving ring-closing metathesis as the key step for the formation of the medium-sized ring.[2,3]

Our interest in carrying out an evaluation of the potential role of calmodulin as a molecular target of the phytotoxic effect of herbarumins I (**1**) and II (**2**) led us to reisolate additional amounts of both substances.[1] During this process, we discovered a new natural product possessing the nonenolide core and one hydroxyl group. According to the previous nomenclature,[1] this substance was named herbarumin III (**3**). Herein, we report its isolation, structure elucidation, and conformational analysis, as well as the effect of the three phytotoxins (**1**−**3**) on bovine-brain calmodulin.



R_1= OH, R_2 = H (**1**)

R_1= R_2= OH (**2**)

R_1= R_2= H (**3**)

Results and Discussion

Phoma herbarum was grown in liquid-substrate fermentation on modified M-D-1. The mycelium and fermentation broth were extracted with EtOAc. The combined extract inhibited radicle growth of seedlings of *A. hypochondriacus* (IC_{50} = 35.5 μg/mL). Bioassay-guided fractionation of the active extract led to the isolation of **3**, (7*R*,9*R*)-7-hydroxy-9-propyl-5-nonen-9-olide, designated with the trivial name of herbarumin III.

The molecular formula of **3** was established as $C_{12}H_{20}O_3$ by HREIMS. The IR as well as the uni- and bidimensional NMR spectra of nonenolide **3** resembled those of herbarumins I (**1**) and II (**2**).[1] This evidence indicated that **3** possessed the same 9-propyl-5-nonen-9-olide core. The most obvious difference between the NMR spectra of **1** and **3** was the absence of signals due to the secondary alcohol functionality at C-8 [δ_H 3.52 (dd, J = 9.8 and 2.5 Hz)/δ_C 73.6] in **1**. In their place, the NMR spectra of **3** exhibited resonances associated with the presence of an additional methylene group [δ_H 1.79 (ddd, J = 14.6, 11.2, and 2.2 Hz) and 1.86 (ddd, J = 14.6, 5.2, and 2.0 Hz)/δ_C 40.5]. Full assignment of the ^{1}H NMR signals of herbarumin III (**3**) were carried out by spectral simulation[4] using the MestRe-C program.[5] This procedure was particularly useful for those hydrogens attached to C-2, C-3, C-4, and C-11, which were found as complex spin systems even at 500 MHz. The stereochemistry and the conformation of **3** in solution was determined by molecular modeling, NOESY data, and comparison of the observed versus the calculated vicinal proton coupling constants of **3** and its epimer at C-9.[6,7] A molecular mechanics study of compound **3** was conducted using a systematic search procedure and the MMX force field calculations[8] as implemented in the PCMODEL program revealing the presence of the global minimum structure at E_{MMX} = 13.88 kcal/mol. This structure was subjected to geometry optimization using a Hartree−Fock/density functional theory hybrid with the 6-31G*/B3LYP level of theory.[9,10] The optimized structure is depicted in Figure 1 ($E_{HF/DFT}$ = −694.98718 hartrees), which was in complete agreement with the correlations observed in the NOESY spectrum. Furthermore, the good correlation between the experimental and calculated coupling constants indicated that the theoretical conformation for **3** is the same as that in solution. Thus, the 10-membered ring in herbarumin III (**3**) exists in a chair−chair−chair conformation,[11] as in the case for herbarumin I (**1**).[1] Additionally, a molecular dynamics study was performed over the minimum energy structure using the leap-frog Verlet algorithm[12] as implemented in the Dynam routine of the PCMODEL program. According to this study, 18 minimum

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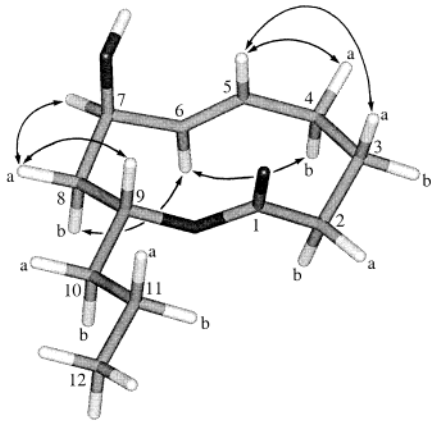

Figure 1. HF/DFT (6-31G*/B3LYP) minimum energy structure of herbarumin III (**3**) showing relevant NOESY correlations.

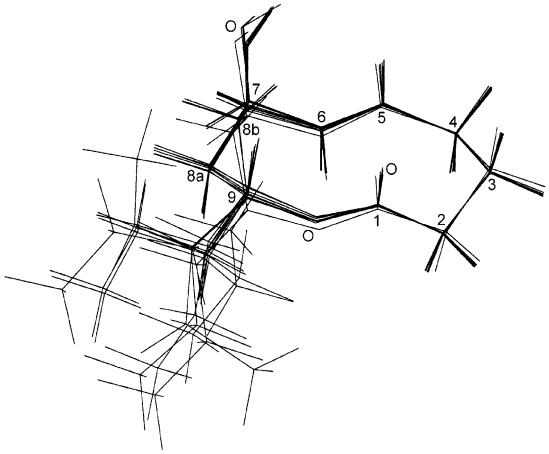

Figure 2. Molecular dynamics samplings for herbarumin III (**3**).

energy conformations were detected for compound **3** within a E_{MMX} range from 13.88 to 18.50 kcal/mol (Figure 2). These structures arose from two conformational features, the rotation of the side chain, which provides nine rotameric species, and a conformational variation in the C-7, C-8, C-9 moiety. The minimum energy structure, which possesses the lactone ring in a boat−chair−chair conformation and the hydroxyl group in a pseudoequatorial orientation, was located at $E_{HF/DFT} = -694.98230$ hartrees, i.e., 3.0 kcal/mol above the global minimum. Therefore, the 10-membered ring of herbarumin III (**3**) essentially remains in a single conformation.

The CD spectrum of compound **3** showed a negative Cotton effect at 219 nm ($\Delta\epsilon = -1.37 \times 10^4$), which was very similar to the transition for the lactone chromophore in herbarumins I (**1**) and II (**2**).[1] Given that the main conformation of the 10-membered ring in the three nonenolides is the same, their absolute configuration must be the same as expected on biogenetic grounds. Therefore, the stereochemical descriptors for the chiral centers of **3** are 7*R*,9*R*.

Herbarumin III (**3**) showed relevant phytotoxic effects when tested against seedlings of *A. hypochondriacus* using the Petri dish bioassay.[13] The lactone ($IC_{50} = 2 \times 10^{-5}$ M) inhibited radicle growth with higher potency than 2,2-dichlorophenoxyacetic acid [2,4-D ($IC_{50} = 2 \times 10^{-4}$ M)], used as positive control. In turn, the phytotoxic effect of **3** was comparable to that of **1** and higher than that of **2**.[1] These results seem to indicate that hydroxylation of the lactone core at C-2 decreases the phytotoxic activity.

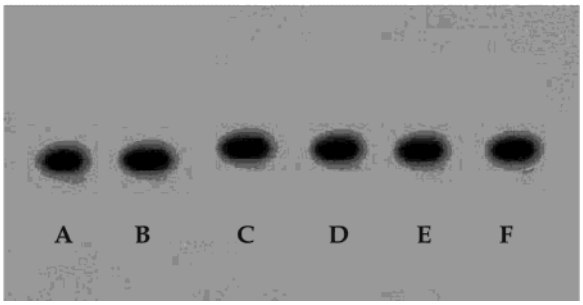

Figure 3. SDS-PAGE of bovine-brain calmodulin after treatment with herbarumins I−III (**1**−**3**). Electrophoresis of 2 μg samples of calmodulin in the presence of 1 mM $CaCl_2$. Pretreatments of the calmodulin samples: 1.5 h at 30 °C in the presence of $CaCl_2$ (A); DMSO (B); chlorpromazine in DMSO (C); **3** (D); **1** (E); and **2** (F). In all cases 0.033 μg/mL of treatment (**1**−**3**) in DMSO was applied.

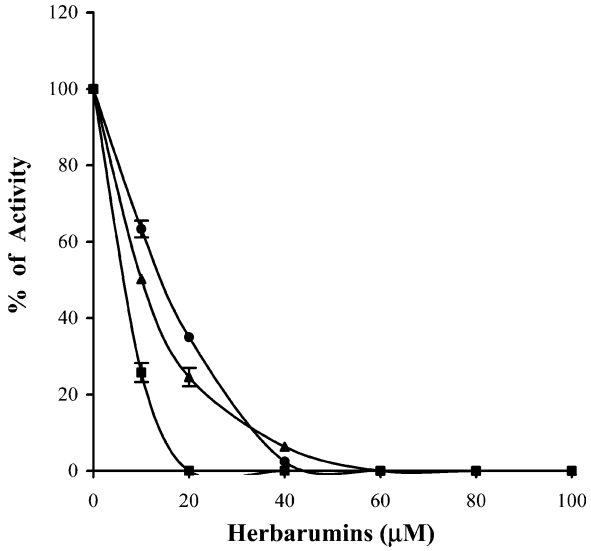

Figure 4. Effect of herbarumins I (●) and II (■) and chlorpromazine (▲) on calmodulin-dependent cAMP. Enzyme activity was measured as a function of compound concentrations at saturating concentrations of bovine-brain calmodulin (0.2 μg). The values are expressed as a percentage of maximum activity obtained with compounds. Each point represents the mean of analyses of three independent biological samples. Vertical bars represent maximum standard deviation.

Compounds **1**−**3** interacted with bovine brain-calmodulin as detected in a SDS-PAGE electrophoresis[14−16] (Figure 3). Calmodulin treated with the lactones had lower electrophoretic mobility than untreated calmodulin. The effect was comparable to that of chlorpromazine, a well-known calmodulin inhibitor. In addition, different concentrations of compounds **1** and **2** inhibited calmodulin-dependent cyclic nucleotide phosphodiesterase (cAMP)[16] as shown in Figure 4. The inhibitory activity of herbarumins I (IC_{50} = 14.2 μM) and II (IC_{50} = 6.6 μM) was higher than that of chlorpromazine (IC_{50} = 9.8 μM). It is important to point out that neither the basal activity of the enzyme nor the calmodulin-independent form of the enzyme was inhibited by the fungal metabolites. The enzyme-inhibition studies suggest that herbarumins I and II may interact with calmodulin to render it inactive. Thus, compounds **1**−**3** are calmodulin inhibitors and should have physiological effects of agrochemical and medicinal interest.

Experimental Section

General Experimental Procedures. Melting points were determined on a Fisher-Johns apparatus and are uncorrected. IR spectra were obtained using KBr disks on a Perkin-Elmer FT 1605 spectrophotometer. UV spectra were obtained on a

Lambda II UV spectrometer in MeOH solution. Optical rotations were taken on a Perkin-Elmer 241 polarimeter. CD spectra were performed on a JASCO 720 spectropolarimeter at 25 °C in MeOH solution. NMR spectra including COSY spectra, and NOESY, HMBC, and HMQC experiments were recorded on a Bruker DMX500, in $CDCl_3$, either at 500 (^1H) or 125 (^{13}C) MHz, using tetramethylsilane (TMS) as an internal standard. EI mass spectra were performed using a JEOL SX 102 mass spectrometer. Open column chromatography: silica gel 60 (70−230 mesh, Merck) and Sephadex LH-20 (Pharmacia). Analytical and preparative TLC were performed on precoated silica gel 60 F254 plates (Merck).

Fungal Material. The fungus *Phoma herbarum* was isolated from a special variety of *Zea mays* ("maíz cacahuacintle") grown in Michoacán, México, in 1998. Cultures of the isolate are maintained in the mycological collection of Laboratorio de Micopatología, Instituto de Biología, UNAM (Voucher: TOX-01020). Stock cultures of the fungus were stored on agar slants of potato dextrose agar (PDA), with subculturing at monthly intervals.

Ten 1.8 L Fernbach flasks, each containing 1 L of modified M-D-I medium that had been sterilized at 120 °C for 15 min and then cooled to room temperature, were individually inoculated with one 1 cm^2 agar plug taken from a stock culture of *P. herbarum* maintained at 4 °C on PDA. Flask cultures were incubated at 28 °C and aerated by agitation on an orbital shaker at 120 rpm for 15 days.

Extraction and Isolation. After incubation, the contents of all flasks were combined and filtered. The culture filtrate (10 L) was extracted exhaustively with EtOAc (3 × 10 L). The combined organic phase was filtered over anhydrous Na_2SO_4 and concentrated in vacuo to give a brown oil (0.70 g). The mycelium was extracted with EtOAc (3 × 1.5 L). The mycelial extract was evaporated to yield 0.372 g of dark brown oil. The combined mycelial and broth extracts (1.072 g) were subjected to open column chromatography on Sephadex LH-20 (2.5 × 60 cm) eluting with a gradient of CH_2Cl_2−MeOH (1:0 → 0:1). Altogether 67 fractions (250 mL each) were collected and pooled on the basis of their TLC profiles to yield six major fractions (FI−FVI). Bioactivity in the phytogrowth bioautographic bioassay (BPIB) showed one active pool (FIII). The active fraction FIII (IC_{50} = 39.4 μg/mL), eluted with CH_2Cl_2−MeOH (9:1), was rechromatographed on Sephadex LH-20 (2.5 × 32 cm) eluting with a gradient of increasing polarity of CH_2Cl_2−MeOH (1:0 → 0:1). Four secondary fractions were obtained (FIII-1−FIII-4). The phytotoxic activity (BPIB) was found in secondary fraction FIII-2 eluted with CH_2Cl_2−MeOH (95:5). Finally, fraction FIII-2 was resolved by preparative TLC [CH_2Cl_2−CH_3OH (90:10)] to render compounds **1** (17.0 mg), **2** (6.0 mg), and **3** (4.0 mg).

Herbarumin III (3): yellow oil, $[\alpha]_D$ +22.0° (*c* 1 mg/mL, EtOH); UV (EtOH) λ_{max} (log ϵ) 209 (314) nm; CD (EtOH) $\Delta\epsilon$ (nm) 2.31 × 10^4 (205), −1.37 × 10^4 (220); IR ν_{max} (KBr) 3390, 2957, 2926, 1721, 1191, 1046 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 5.61 (1H, ddd, *J* = 15.8, 4.0, 1.5 Hz, H-6), 5.47 (1H, dddd, *J* = 15.8, 12.0, 4.6, 2.3 Hz, H-5), 5.27 (1H, dddd, *J* = 11.2, 8.5, 4.2, 2.0 Hz, H-9), 4.42 (1H, ddddd, *J* = 5.2, 4.0, 2.3, 2.2, 1.7 Hz, H-7), 2.37 (1H, ddddddd, *J* = 14.0, 4.6, 3.5, 3.3, 1.7, 1.5, 0.4 Hz, H-4a), 2.29 (1H, dddd, *J* = 13.7, 6.2, 1.6, 0.4 Hz, H-2a), 2.02 (1H, ddd, *J* = 13.7, 12.6, 1.5 Hz, H-2b), 1.99 (1H, ddddd, *J* = 13.4, 13.3, 12.6, 3.5, 1.6 Hz, H-3a), 1.98 (1H, dddd, *J* = 14.0, 13.3, 12.0, 4.0 Hz, H-4b), 1.86 (1H, ddd, *J* = 14.6, 5.2, 2.0 Hz, H-8a), 1.79 (1H, ddd, *J* = 14.6, 11.2, 2.2 Hz, H-8b), 1.76 (1H, ddddd, *J* = 13.4, 6.2, 4.0, 3.3, 1.5 Hz, H-3b), 1.55 (1H, dddd, *J* = 13.5, 9.2, 8.5, 4.9 Hz, H-10b), 1.42 (1H, dddd, *J* = 13.5, 9.2, 6.8, 4.2 Hz, H-10a), 1.32 (1H, dddq, *J* = 13.5, 9.2, 7.3, 6.8 Hz, H-11a), 1.32 (1H, dddq, *J* = 13.5, 9.2, 7.3, 4.9 Hz, H-11b), 0.90 (3H, t, *J* = 7.3 Hz, H-12); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 176.8 (C-1), 134.5 (C-6), 124.9 (C-5), 68.0 (C-7), 67.8 (C-9), 40.5 (C-8), 37.4 (C-10), 34.6 (C-2), 33.6 (C-4), 25.9 (C-3), 18.4 (C-11), 13.8 (C-12); EIMS *m/z* 212 [M^+] (5), 194 (35), 169 (20), 151 (73), 143 (30), 125 (100), 113 (47), 97 (70), 63 (56), 70 (53), 55 (78), 41 (48); HREIMS *m/z* 212.1416 (calcd for $C_{12}H_{20}O_3$, 212.1412).

Molecular Modeling Calculations. Minimum energy structures were generated using the MMX force field[8] as implemented in the PCMODEL program V 6.00 (Molecular Modeling Software available from Serena Software, Bloomington, IN). The conformational search for the 10-membered ring of **3** was carried out considering torsion angle rotations of ca. 30°, which were minimized and evaluated taking into account the E_{MMX} convergence criterion. The propyl side-chain was minimized considering all possible alternate conformations. An implicit solvent description was applied using a dielectric constant term of 4.8 ($CHCl_3$). After the minimization procedure, the global minimum structure of **3** served as the starting point for the molecular dynamics analysis, which was performed during 1000 ps, starting with an initial temperature of 5 K and then warmed to 298 K over a period of 300 ps. A time step for numerical integration of 1 fs was employed during all the molecular dynamics processes using a viscosity term of 0.0001 cP. The heat transfer time was set at 1 fs and the equilibration time at 300 fs. Samples were saved every 1 ps, resulting in 1000 structures for the trajectory. The complete structure set was submitted to the batch routine to reminimize all the saved structures, which were analyzed and classified by torsion angles employing a total energy diagram versus time. The molecular mechanics minimum energy structures were geometry optimized using a restricted hybrid Hartree−Fock/density functional theory[9,10] model at the 6-31G*/B3LYP level of theory as implemented in the Spartan'02 program (Wavefunction, Inc., Irvine, CA).

Phytogrowth-Inhibitory Bioassay. The phytogrowth inhibitory activity of the extract, fractions, and **3** was evaluated on seeds of *Amaranthus hypochondriacus* L**.** by using a Petri dish bioassay.[13] The results were analyzed by ANOVA ($P < 0.05$), and IC_{50} values were calculated by Probit analysis based on percent of radicle growth or germination inhibition. The extract and fractions were evaluated at 10, 100, and 1000 μg mL^{-1}. Herbarumin III (**3**) was tested at 1, 10, and 100 μg mL^{-1}. 2,4-D was used as the positive control. The bioassays were performed at 28 °C. In addition, a direct bioautographic bioassay system was employed to guide secondary fractionation and speed up the isolation of any active compound. The direct bioautographic assay was carried out as previously described.[13]

Evaluation of the Interaction of Compounds 1−3 with Bovine-Brain Calmodulin. The interaction of the isolated compounds with bovine-brain calmodulin (SIGMA) was performed using a nondenaturing homogeneous electrophoresis (SDS-PAGE). SDS-PAGE was performed according to a previously described procedure using 15% polyacrylamide gels.[14−16] The interaction of the phytotoxins with calmodulin was evaluated by observing the difference in electrophoretic mobility in the presence of Ca^{2+}. Each electrophoretic run was done in triplicate, and chlorpromazine was used as the positive control. The experimental conditions are described briefly in the legend of Figure 3.

Cyclic Nucleotide Phosphodiesterase Assay. The assay of cyclic nucleotide phosphodiesterase was performed by a modification of the method described by Sharma and Wang.[8,9] Bovine-brain calmodulin (0.2 μg) as enzyme activator was incubated with 0.015 units of calmodulin-deficient-calmodulin-dependent cAMP from bovine brain (SIGMA) for 3 min in 800 μL of assay solution containing 0.3 units 5′-nucleotidase, 45 mM Tris-HCl, 5.6 mM magnesium acetate, 45 mM imidazole, and 2.5 mM calcium chloride, pH 7.0. The test compounds were then added to the assay medium at 10, 20, 40, 60, 80, and 100 μM in DMSO, and the samples were incubated for 30 min. Then 100 μL of 10.8 mM cAMP, pH 7.0, was added to start the assay. After 30 min the assay was stopped by the addition of 100 μL of 55% trichloroacetic acid solution. All the above steps were carried out at 30 °C. The phosphodiesterase reaction was coupled to the 5′-nucleotidase (*Crotalus atrox* venom from SIGMA) reaction, and the amount of inorganic phosphate released represented the activity of the phosphodiesterase. The phosphate produced in the assay was measured by the method of Sumner.[18] The wavelength used for the phosphate assay was 660 nm. Once more, chlorpromazine was

used as the positive control. The experiments to determine the effect on the basal activity of calmodulin-deficient-calmodulin-dependent cAMP and calmodulin-independent cAMP were performed as described above but in the absence of calmodulin. The aim of the last set of experiments was to confirm the calmodulin-dependence of the observed effects.

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