Phytotoxic Compounds from the New Coprophilous Fungus *Guanomyces polythrix*[1]

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Bioactivity-directed fractionation of the fermentation broth and mycelium of the coprophilous fungus *Guanomyces polythrix* led to the isolation of several phytotoxic compounds, including five new naphthopyranone derivatives (**1**−**5**). In addition, rubrofusarin B, emodin, citrinin, and 4-hydroxybenzoic acid methyl ester were obtained. The structures of the new compounds were established by spectral and chiroptical methods. The isolates caused significant inhibition of radicle growth of two weed seedlings (*Amaranthus hypochondriacus* and *Echinochloa crusgalli*) and interacted with both spinach and bovine brain calmodulins.

As a part of our search for potential herbicide agents from natural sources, we describe in this investigation the isolation and structure elucidation of the major phytotoxic principles from the culture broth and mycelium of the new coprophilous fungus *G. polythrix*. In addition, the potential role of calmodulin as molecular target of the phytotoxic action of the isolated compounds was investigated. Calmodulin is a highly conserved calcium-modulated protein that appears to be ubiquitous among eukaryotes. In plants, this protein is a fundamental component of calcium signal transduction pathways during germination and plant growth. It modulates the activity of several important plant enzymes such as NAD-kinase, glutamate decarboxylase, and Ca^{2+}-ATPase.[2−4] Plant calmodulins share many structural and functional features with their homologues from animals, but the expression of multiple protein isoforms appears to be a distinctive feature of higher plants. Therefore, calmodulin and the proteins it regulates could be important targets for the action of many phytotoxins. In this context, it has been previously described that some fungal phytotoxins such as ophiobolin A[5,6] and several plant aromatic compounds[7,8] interact with calmodulin and inhibit its enzyme activator properties.

G. polythrix is a new species of coprophilous fungus related to the genus *Chaetomium* (Chaetomiaceae). Morphological, physiological, and molecular studies on this fungus revealed that it belongs to a new genus. Its description will be published soon.[9] The new fungus was isolated in 1979, from bat guano obtained from zone III of the cave "Cueva del Diablo" or "Ocotlitlán", in Tepozotlán, Morelos, México.

Results and Discussion

The new fungus *G. polythrix* was grown in liquid−substrate fermentation on potato dextrose broth (PDB). The culture broth and the mycelium were extracted with CH_2Cl_2. The combined extract showed phytotoxic activity (Table 1) when evaluated on seedlings of *Amaranthus hypochondriacus* L. and *Echinochloa crusgalli* (L.) Beauv. using a Petri dish bioassay.[10] Bioactivity-guided fractionation of this extract led to the isolation of nine phytotoxins (see Experimental Section). Compounds **1**−**5** are new natural products and were characterized by spectroscopic and chiroptical methods as (2*S*,3*S*)-5-hydroxy-6,8-dimethoxy-2,3-dimethyl-4*H*-2,3-dihydronaphtho[2,3-*b*]-pyran-4-one (**1**); (2*S*,3*S*)-5-hydroxy-6,8,10-trimethoxy-2,3-dimethyl-4*H*-2,3-dihydronaphtho[2,3-*b*]-pyran-4-one (**2**); (2*S*)-5-hydroxy-6,8-dimethoxy-2-methyl-4*H*-2,3-dihydronaphtho[2,3-*b*]-pyran-4-one (**3**); (2*S*)-5-hydroxy-6,8,10-trimethoxy-2-methyl-4*H*-2,3-dihydronaphtho[2,3-*b*]-pyran-4-one (**4**); and 5-hydroxy-6,8-dimethoxy-2,3-dimethyl-4*H*-naphtho[2,3-*b*]-pyran-4-one (**5**). In addition, the known compounds rubrofusarin B (**6**),[11−13] emodin (**7**),[14,15] citrinin (**8**),[16,17] and 4-hydroxybenzoic acid methyl ester (**9**)[18] were obtained. The spectral properties of the known compounds, including IR, ^{1}H NMR, and ^{13}C NMR data, were identical to those previously described in the literature.

Table 1. Phytogrowth-Inhibitory Activity of the CH_2Cl_2 Extract and Isolated Compounds from Coprophilous Fungus on Radicle Elongation (IC_{50}, M) of *A. hypochondriacus* and *E. crusgalli*

compound	*A. hypochondriacus*	*E. crusgalli*
extract	54.3[a]	384.3[a]
1	1.0×10^{-4}	1.2×10^{-4}
2	6.5×10^{-5}	6.1×10^{-5}
3	2.3×10^{-5}	8.7×10^{-5}
4	1.3×10^{-5}	4.0×10^{-5}
5	8.0×10^{-5}	1.7×10^{-4}
6	1.3×10^{-5}	8.7×10^{-5}
7	1.3×10^{-4}	2.0×10^{-5}
8	5.5×10^{-5}	6.3×10^{-5}
9	2.3×10^{-5}	6.3×10^{-4}
2,4-D[b]	1.8×10^{-4}	8.8×10^{-4}

[a] Expressed in μg/mL. [b] Positive control (2,4-dichlorophenoxyacetic acid).

Compound **1** has a molecular formula of $C_{17}H_{18}O_5$ (nine unsaturations) as inferred from HREIMS and ^{13}C NMR. The NMR spectra (Table 2) were similar to those of other 2,3-dihydronaphthopyran derivatives.[19] These spectra demonstrated the presence of a carbonyl group; a chelated hydroxyl functionality; two methoxyl groups; two *m*-related and one isolated aromatic protons; two methyl groups, each attached to an aliphatic methine; and several nonprotonated sp^2 carbons. From the ^{1}H−^{1}H COSY spectrum, the methines [δ_H/δ_C 4.20 (dq, J = 10.5, 6.5 Hz, H-2) and 2.65

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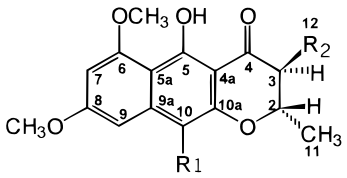
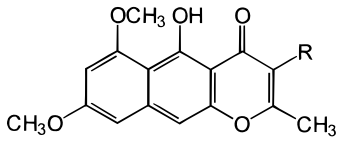
Table 2. NMR Data for (2*S*,3*S*)-5-Hydroxy-6,8-dimethoxy-2,3-dimethyl-2,3-dihydro-4*H*-naphtho[2,3-*b*]-pyran-4-one (**1**)[a]

position	^{13}C (δ)	^{1}H (δ, mult., *J* in Hz)	NOESY	HMBC
1				
2	78.01	4.20 (dq, 6.5, 10.0)	11-CH_3, 3-H, 12- CH_3	3, 11- CH_3
3	46.43	2.65 (dq, 6.5, 10.0)	12-CH_3, 2-H, 11- CH_3	2, 12- CH_3
4	200.06			2, 3, 12- CH_3
4a	102.99			3,OH-5,10
5	165.11			OH-5
5a	107.17			OH-5, 7, 9, 10
6	161.28			7, 6-OCH_3
7	96.39	6.27 (d, 2.5)	9, 6-OCH_3	9
8	162.30			7, 8-OCH_3, 9
9	98.27	6.43 (d, 2.5)	7, 10, 8-OCH_3	7, 10
9a	143.30			9, 10
10	101.22	6.48 (s)	9	9
10a	156.00			2, 10
CH_3−11	19.75	1.50 (d, 6.5)	2-H, 3-H, 12- CH_3	2, 3
CH_3−12	10.44	1.26 (d, 7.0)	3-H, 2-H, 11- CH_3, 5-OH	3, 2
OCH_3−6	56.00	3.96 (s)	7	
OCH_3−8	55.34	3.88 (s)	9	
OH-5		14.49 (s)	12-CH_3	

[a] Spectra were recorderd in $CDCl_3$ (^{1}H, 500 MHz and ^{13}C, 125 MHz).



1 R_1= H, R_2= CH_3
2 R_1= CH_3O, R_2= CH_3
3 R_1= H, R_2= H
4 R_1= CH_3O, R_2= H



5 R= CH_3
6 R= H

(dq, J = 10.5, 6.5 Hz, H-3)/78.0 and 46.4] were adjacent, and the coupling constant value (J = 10.5 Hz) indicated a *trans*-diaxial relationship. Cursory inspection of the NOESY and HMBC spectra (see Table 2) indicated the position of the methoxy, hydroxy, and methyl groups on the dihydronaphthopyranone skeleton. The application of chiroptical methods for the assignment of the absolute configuration of benzo-γ-dihydropyrones such as flavanones and 4-hydroxyflavanones is well documented.[20] In flavanones, the configuration 2*S* shows a positive Cotton effect around 330 nm; conversely, the configuration 2*R* shows a negative Cotton effect in the same region. The presence of a trans hydroxy group at C-3 does not modify the relation between the sign and configuration. Thus, the stereogenic centers C-2 and C-3 were each determined to have the *S* absolute configuration on the basis of the negative Cotton effect around 314 nm in the CD spectrum.[20]

Compound **2** had the composition $C_{18}H_{20}O_6$ (nine unsaturations) as determined by HREIMS and ^{13}C NMR, differing from **1** by 30 mass units. This observation, as well as the NMR data (Table 3), suggested that **2** was the C-10 methoxy derivative of **1**. The NMR spectra of **2** were almost identical with those of **1**, except for the presence of an additional methoxyl signal (δ_H/δ_C 3.89/60.6) in place of the isolated aromatic proton signal. As in the case of compound **1**, the disposition of the substituents along the 2,3-dihydronaphthopyranone nucleus was established by the analysis of the NOESY and HMBC correlations (see Table 3). The strong interactions observed both in the ^{1}H−^{1}H COSY and NOESY spectra between the signal at δ_H 6.91 (H-9) and those at δ_H 3.94 and 3.89 (CH_3O-8 and CH_3O-10, respectively) were consistent with the placement of the third methoxyl group at C-10. Once more, the absolute stereochemistry at C-2 and C-3 was assigned on the basis of the negative Cotton effect around 314 nm in the CD spectrum.[20] Consequently, the structure of compound **2** was determined to be (2*S*,3*S*)-5-hydroxy-6,8,10-trimethoxy-2,3-dimethyl-4*H*-dihydronaphtho[2,3-*b*]-pyran-4-one.

The HREIMS of compounds **3** and **4** showed the molecular ions at *m*/*z* 288.0992 and 318.1108, respectively, and, together with the ^{13}C NMR, indicated the molecular formulas of $C_{16}H_{16}O_5$ and $C_{17}H_{18}O_6$, respectively. The NMR and CD characteristics of **3** and **4** (see Experimental Section) were highly similar to those of **1** and **2**, respectively. In the NMR spectra of **3** and **4**, signals attributable to a methylene group replaced those corresponding to the methyl and methine groups at C-3 of **1** and **2**. In addition, a marked difference in chemical shift for H-2 (δ_H 4.60 in both cases) was observed. The CD spectra of both compounds again showed a negative Cotton effect around 314 nm, supporting the depicted 2*S* absolute configuration. These observations, in conjuction with the data obtained using the same array of NMR techniques as in the structure determination of **1** and **2**, allowed the identification of compounds **3** and **4** as (2*S*)-5-hydroxy-6,8-dimethoxy-2-methyl-4*H*-2,3-dihydronaphtho[2,3-*b*]-pyran-4-one and (2*S*)-5-hydroxy-6,8,10-trimethoxy-2-methyl-4*H*-2,3-dihydronaphtho [2,3-*b*]-pyran-4-one, respectively.

HREIMS and NMR data for compound **5** indicated a molecular formula of $C_{17}H_{18}O_6$ (ten unsaturations) differing from **1** by two mass units. This observation, as well as the NMR spectra (see Experimental Section), suggested that **5** was the 2,3-dehydro derivative of **1**. The most obvious difference between the NMR spectra of **5** and **1** is the presence of two nonprotonated vinylic signals (δ_C 167.4 and 119.2) in **5**, instead of the resonances attributed to H-2/C-2 and H-3/C-3 in **1**. In addition, the chemical shift values for the methyl groups on the pyrone ring were shifted paramagnetically in comparison to those in **1**. Thus, the structure of compound **5** was established as 5-hydroxy-6,8-dimethoxy-2,3-dimethyl-4*H*-naphtho[2,3-*b*]-pyran-4-one.

Natural products **1**−**9** were evaluated for their ability to inhibit seed germination and seedling growth of *A. hypochondriacus* and *E. crusgalli*. Table 1 summarizes the

Table 3. NMR Data for (2*S*,3*S*)-5-Hydroxy-6,8,10-trimethoxy-2,3-dimethyl-2,3-dihydro-4*H*-naphtho[2,3-*b*]-pyran-4-one (**2**)[a]

position	^{13}C (δ)	^{1}H (δ, mult., *J* in Hz)	NOESY	HMBC
1				
2	78.32	4.28 (dq, 6.5, 10.0)	11-CH_3, 3-H, 12- CH_3	3, 11- CH_3
3	46.54	2.68 (dq, 7.0, 10.0)	12-CH_3, 2-H, 11- CH_3	2, 12- CH_3
4	200.09			2, 3, 12- CH_3
4a	102.65			3,OH-5,10
5	161.63			OH-5
5a	106.95			OH-5, 7, 9
6	161.20			7, 6-OCH_3
7	92.59	6.34 (d, 2.5)	9, 6-OCH_3	9
8	162.68			7, 8-OCH_3, 9
9	96.78	6.91 (d, 2.5)	7, 10, 8-OCH_3, 10-OCH_3	7
9a	131.83			9
10	162.68			9
10a	138.27			2, 10
CH_3−11	19.85	1.57 (d, 6.5)	2-H, 3-H, 12- CH_3	2, 3
CH_3−12	10.75	1.28 (d, 7.0)	3-H, 2-H, 11- CH_3, 5-OH	3, 2
OCH_3−6	56.15	3.97 (s)	7	
OCH_3−8	55.48	3.94 (s)	9	
OCH_3−10	60.63	3.89 (s)	9	
OH-5		14.30 (s)	12-CH_3	

[a] Spectra were recorderd in $CDCl_3$ (^{1}H, 500 MHz and ^{13}C, 125 MHz).

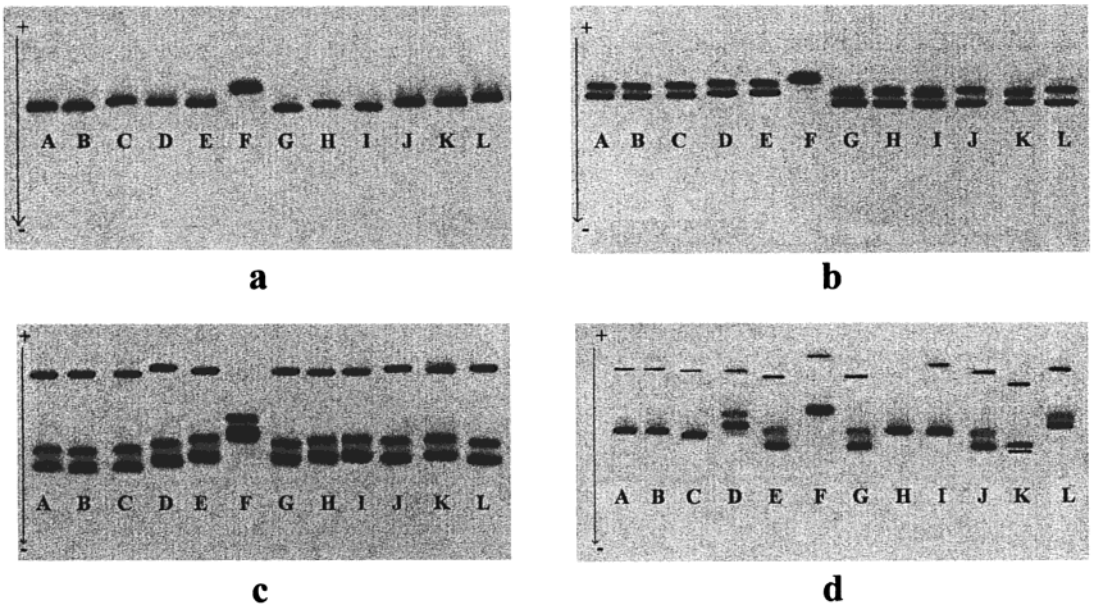


Figure 1. SDS−PAGE of bovine (a and b) and spinach (c and d) calmodulins after treatment with compounds **1**−**9**. Electrophoresis of 2-μg samples of calmodulins in the presence of 1 mM $CaCl_2$ (a and c) or EGTA (b and d). Pretreatements of the calmodulin samples, for 1.5 h at 30 °C in the presence of $CaCl_2$ (a and c) or EGTA (b and d). A, no additions; B, DMSO; C, 0.033 μg/mL quercetin in DMSO; D, **1**; E, **3**; F, **2**; G, **4**; H, **5**; I, **6**; J, **7**; K, **8**; L, **9**. In all cases 0.033 μg/mL of treatment (**1**−**9**) in DMSO were applied.

phytotoxic effects of the isolates on seedling growth. The results are expressed as IC_{50} values (50% inhibitory concentration). In general, the tested compounds showed significant phytotoxic effect and were more potent as radicle growth inhibitors than the positive control [2,4-dichlorophenoxyacetic acid (2,4-D)]. The nine compounds reduced the radicle growth of both target species in a concentration-dependent manner. Compounds **3**, **4**, **6**, and **9** were the most active against *A. hypochondricus*; however, *E. crusgalli* was more affected by emodin.

In an attempt to discover potential herbicides with an effect on the protein calmodulin, the phytotoxic compounds were further evaluated for their ability to bind spinach and bovine brain calmodulin-Ca^{2+} complex. The interaction of any compound with calmodulin-Ca^{2+} complex alters its conformation and changes its electrophoretic mobility as detected in a SDS−PAGE.[5,6] Figure 1 shows that bovine calmodulin treated with Ca^{2+} (1 mM) and the isolates (0.033 μg/mL) has a lower electrophoretic mobility than untreated calmodulin. The strongest effect was observed with compound **2**. The inhibition of bovine calmodulin induced by compounds **1** and **3**−**9** seems to be dependent on Ca^{2+}. Phytototoxins **1** and **3**−**9** in the presence of Ca^{2+} retarded the electrophoretic mobility of calmodulin. Upon conducting the same assay in the presence of EGTA (ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid), a calcium-chelating agent, the mobility of calmodulin is not retarded (Figure 1). In the case of compound **2**, however, the effect seems to be independent of Ca^{2+} because in the presence of EGTA the retardation on the electrophoretic mobility of calmodulin provoked by this compound was the same as in the presence of Ca^{2+}. Because the isolates are phytotoxins, their effects on a plant calmodulin were investigated. Spinach calmodulin was also found to interact with compounds **1**−**9**. Once again, compound **2** demonstrated the stronger interaction, and its effect was independent of Ca^{2+} (Figure 1).

In conclusion, the results of this study showed that the compounds isolated from the fungus *G. polythrix* are calmodulin-Ca^{2+} inhibitors and possess promising phyto-

growth inhibitory activity. Further work is in progress to determine the nature (covalent vs noncovalent) of the interaction of compound **2** with calmodulin.

Experimental Section

General Experimental Procedures. Melting points were determined in a Fisher-Johns apparatus and are uncorrected. IR spectra were obtained using KBr disks on a Perkin-Elmer 599 B spectrophotometer. UV spectra were obtained on a Shimadzu 160 UV spectrometer in MeOH solution. Optical rotations were recorded on a JASCO DIP 360 digital polarimeter. CD spectra were performed on a JASCO 720 spectropolarimeter at 25 °C in MeOH solution. NMR spectra, including COSY spectra, NOESY, HMBC, and HMQC experiments were recorded on a Varian UNITY PLUS 500 spectrometer in $CDCl_3$ or $CDCl_3$−DMSO-d_6 either at 500 MHz (^{1}H) or 125 MHz (^{13}C), using tetramethylsilane (TMS) as an internal standard. EIMS were performed using a JEOL JMS-AX505 HA spectrometer, at an ionization energy of 70 eV. Column chromatography: Si gel 60 (70−230 mesh, Merck). Analytical and preparative TLC were performed on precoated Si gel 60 F_{254} plates (Merck).

Fungal Material. The fungus *G. polythrix* was isolated from bat guano, obtained from zone III of the cave "Cueva del Diablo" or "Ocotlitlán", in Tepozotlán, Morelos, México, in 1979. Since then, cultures of the fungus have been preserved lyophilized in liquid nitrogen. Subcultures were obtained in several culture media, such as PDA, V8 agar, and others. Cultures of this new fungus are maintained at the National Herbarium (MEXU, voucher: 24486), Instituto de Biología, UNAM, México, and in the mycological collection of the Department of Plant Pathology (voucher: GAM 5803), University of Georgia, Athens, Georgia.

Thirty 2-L Erlenmeyer flasks, each containing 400 mL of PDB (Difco), were individually inoculated with one 1-cm^2 agar plug taken from a stock culture of *G. polythrix* maintained at 4 °C on potato dextrose agar. Flask cultures were incubated at 28 °C and aerated by agitation on an orbital shaker at 150 rpm for 15 days.

Extration and Isolation of 1−9. After incubation, the flask contents were combined and filtered. The culture filtrate (12 L) was extracted exhaustively with CH_2Cl_2 (3 × 12 L). The combined organic phase was filtered over anhydrous Na_2SO_4 and concentrated in vacuo to give a green solid (1.4 g). In addition, the mycelium was extracted with CH_2Cl_2 (3 × 2 L). The combined mycelial extract was evaporated to yield 5.7 g of a green solid. The extracts (culture and mycelial) were combined (7.1 g) and subjected to Si gel (200 g) column chromatography eluting with a gradient of hexane−CH_2Cl_2 (5:5→0:10) and CH_2Cl_2−MeOH (9.9:0.1→0:10). In all, 465 fractions (50 mL each) were collected and pooled on the basis of their TLC profiles to yield 12 mayor fractions (FI−FXII). Bioactivity in the bioautographic bioassay showed five active pools: FIV (113.6 mg), FV (164.2 mg), FVI (316.3 mg), FVII (143.1 mg), and FVIII (262.5 mg). From the active fraction FIV, eluted with hexane−CH_2Cl_2 (2:8), crystallized 9.2 mg of compound **1**. From fraction FV eluted with hexane−CH_2Cl_2 (1:9), cocrystallized a mixture (53 mg) of **1** and **2**. The mixture was resolved by preparative TLC (CH_2Cl_2) to yield **1** (20 mg) and **2** (10.5 mg). The mother liquors from phytotoxic fraction FV (164.2 mg) were rechromatographed on a Si gel (15 g) column eluting with a gradient of hexane−CH_2Cl_2 (5:5→0:10) and CH_2Cl_2−MeOH (9.9:0.1→5:5). Ten secondary fractions were obtained (FV-1−FV-10). According to the bioautographic bioassay, the phytotoxic activity was concentrated in secondary fraction FV-5. Extensive TLC (CH_2Cl_2) of fraction FV-5 yielded **3** (9.0 mg) and **4** (5 mg). Primary fraction FVI (316.3 mg), eluted with CH_2Cl_2, was further resolved on another Si gel (30 g) column using the same elution system as for fraction FV to yield eight secondary fractions (FVI-1−FVI-8). The phytotoxic activity was found in secondary fraction FVI-6, eluted with CH_2Cl_2−MeOH (99:1). Further purification of this fraction by preparative TLC (CH_2Cl_2) yielded **6** (7.1 mg), **5** (7 mg), and **9** (8 mg). Active fraction FVII (143.1 mg), eluted with CH_2Cl_2−MeOH (99:1), was rechromatographed on a Si gel column using the solvent gradient systems of hexane−CH_2Cl_2 (5:5→0:10) and CH_2Cl_2−MeOH (9.9:0.1→0:10). In all, 10 secondary fractions were obtained (FVII-1−FVII-10), the phytotoxic activity being in fraction FVII-7. From this fraction crystallized spontaneously 9.5 mg of **7**. Finally, from the phytotoxic fraction FVIII crystallized 55 mg of compound **8**.

(2*S*,3*S*)-5-Hydroxy-6,8-dimethoxy-2,3-dimethyl-4*H*-2,3-dihydronaphtho[2,3-*b*]-pyran-4-one (1): yellow crystalline needles, mp 165−167 °C; $[\alpha]_D$ +128° (*c* 1 mg/mL MeOH); UV (MeOH) λ_{max} (log ϵ) 409.5 (3.85), 320 (3.97), 277.5 (4.54), 252 (4.19), 235 (4.43), 214 (4.26), 209 (4.27) nm; CD (MeOH) $\Delta\epsilon$ (nm) -1.5×10^4 (314); IR ν_{max} (KBr) 3500, 1638, 1610 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 2; EIMS *m/z* 302 [M$^+$ (100)], 246 (90), 218 (12), 203 (6), 190 (19); HRMS *m/z* 302.1159 (calcd for $C_{17}H_{18}O_5$, 302.1154).

(2*S*,3*S*)-5-Hydroxy-6,8,10-trimethoxy-2,3-dimethyl-4*H*-2,3-dihydronaphtho[2,3-*b*]-pyran-4-one (2): yellow solid, mp 153−155 °C; $[\alpha]_D$ +150° (*c* 1 mg/mL MeOH); UV (MeOH) λ_{max} (log ϵ) 411 (3.34), 320 (3.46), 277.5 (4.07), 252 (3.71), 235 (3.96), 214 (4.28), 209 (4.29) nm; CD (MeOH) $\Delta\epsilon$ (nm) -1.6×10^4 (314); IR ν_{max} (KBr) 3500, 1638, 1610 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 3; EIMS *m/z* 332 [M$^+$ (98)], 317 (90), 261 (100), 233 (66), 218 (14); HRMS *m/z* 332.1255 (calcd for $C_{18}H_{20}O_6$, 332.1259).

(2*S*)-5-Hydroxy-6,8-dimethoxy-2-methyl-4*H*-2,3-dihydronaphtho[2,3-*b*]-pyran-4-one (3): pale yellow needles, mp 175−177 °C; $[\alpha]_D$ +87.5° (*c* 1 mg/mL MeOH); UV (MeOH) λ_{max} (log ϵ) 401.5 (4.10), 331 (4.23), 318 (4.19), 278 (4.83), 232 (4.69), 223 (4.16), 247 (4.46); CD (MeOH) $\Delta\epsilon$ (nm) -1.5×10^4 (312); IR ν_{max} (KBr) 3500, 1639 and 1611 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 14.46 (1H, s, OH-5), 6.52 (1H, s, H-10), 6.45 (1H, d, J = 2.5 Hz, H-9), 6.30 (1H, d, J = 2.5 Hz, H-7), 4.58 (1H, dqd, J = 11.0, 6.25, 3.5 Hz, H-2), 3.97 (3H, s, OCH_3-6), 3.89 (3H, s, OCH_3-8), 2.81 (1H, dd, J = 17.0, 11.0 Hz, H-3$_b$), 2.73 (1H, dd, J = 17.0, 3.5 Hz, H-3$_a$), 1.51 (3H, d, J = 6.5 Hz, CH_3-11); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 197.60 (C-4), 165.31 (C-5), 162.48 (C-8), 161.46 (C-6), 156.21 (C-10a), 143.50 (C-9a), 107.39 (C-5a), 103.13 (C-4a), 101.41 (C-10), 98.49 (C-9), 96.58 (C-7), 73.16 (C-2), 56.06 (OCH_3-6), 55.38 (OCH_3-8), 43.86 (C-3), 21.16 (CH_3-11); EIMS *m/z* 288 [M$^+$ (100)], 246 (50), 218 (10), 190 (20); HRMS *m/z* 288.0992 (calcd for $C_{16}H_{16}O_5$, 288.0997).

(2*S*)-5-Hydroxy-6,8,10-trimethoxy-2-methyl-4*H*-2,3-dihydronaphtho[2,3-*b*]-pyran-4-one (4): pale yellow needles, mp 160−162 °C; $[\alpha]_D$ +145.8° (*c* 1 mg/mL MeOH); UV (MeOH) λ_{max} (log ϵ) 413 (3.14), 332 (3.17), 320.5 (3.24), 277 (3.85), 235.5 (3.74), 214 (3.52), 207 (3.55); CD (MeOH) $\Delta\epsilon$ (nm) -1.5×10^4 (313); IR ν_{max} (KBr) 3490, 1640, and 1610 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 14.21 (1H, s, OH-5), 6.92 (1H, d, J = 2.5 Hz, H-9), 6.35 (1H, d, J = 2.5 Hz, H-7), 4.60 (1H, dqd, J = 10.5, 6.25, 4.25 Hz, H-2), 3.97 (3H, s, OCH_3-6), 3.95 (3H, s, OCH_3-8), 3.89 (3H, s, OCH_3-10), 2.80 (1H, dd, J = 17.0, 10.5 Hz, H-3$_b$), 2.75 (1H, dd, J = 17.0, 4.25 Hz, H-3$_a$), 1.57 (3H, d, J = 6.5 Hz, CH_3-11); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 197.65 (C-4), 162.88 (C-8), 162.88 (C-10), 161.83 (C-5), 161.36 (C-6), 138.45 (C-10a), 132.03 (C-9a), 107.10 (C-5a), 103.36 (C-4a), 96.90 (C-9), 92.76 (C-7), 73.42 (C-2), 60.66 (OCH_3-10), 56.15 (OCH_3-6), 55.49 (OCH_3-8), 43.97 (C-3), 21.00 (CH_3-11); EIMS *m/z* 288 [M$^+$ (100)], 246 (50), 218 (10), 190 (20); EIMS *m/z* 318 [M$^+$ (98)], 303 (90), 261 (100), 233 (45), 218 (20); HRMS *m/z* 318.1108 (calcd for $C_{17}H_{18}O_6$, 318.1103).

5-Hydroxy-6,8-dimethoxy-2,3-dimethyl-4*H*-naphtho-[2,3-*b*]-pyran-4-one (5): pale yellow needles, mp 195−197 °C; UV (MeOH) λ_{max} (log ϵ) 398.5 (3.47), 274 (3.64), 237.5 (3.18), 224.5 (3.46); IR ν_{max} (KBr) 3148, 1732, and 1650 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 15.22 (1H, s, OH-5), 6.93 (1H, s, H-10), 6.57 (1H, d, J = 2.4 Hz, H-9), 6.37 (1H, d, J = 2.4 Hz, H-7), 4.00 (3H, s, OCH_3-6), 3.92 (3H, s, OCH_3-8), 2.39 (3H, s, CH_3-11) 2.03 (3H, s, CH_3-12); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 184.13 (C-4), 167.40 (C-2), 162.61 (C-8), 161.50 (C-6), 160.62 (C-5), 153.30 (C-10a), 141.09 (C-9a), 119.22 (C-3), 108.40 (C-5a), 104.21 (C-4a), 101.04 (C-10), 97.80 (C-9), 97.23 (C-7), 56.08 (OCH_3-6), 55.40 (OCH_3-8), 23.12 (CH_3-11) 20.68 (CH_3-12);

EIMS *m*/*z* 300 [M^+ (100)], 282 (25), 271 (65), 254 (15), 229 (12), HRMS *m*/*z* 300.0994 (calcd for $C_{17}H_{16}O_5$, 300.0997).

Phytogrowth-Inhibitory Bioassays. The phytogrowth inhibitory activity of the extract and pure compounds was evaluated on seeds of *A. hypochondriacus* and *E. crusgalli* by using a Petri dish bioassay.[10] In addition, a direct bioautographic bioassay system was employed to guide secondary fractionation and speed up the isolation of active compounds. The direct bioautographic assay was carried out as previously described.[10] 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 was evaluated at 10, 100, and 1000 μg mL^{-1}. The pure compounds were 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.

Evaluation of the Interaction of Compounds 1−9 with Spinach and Bovine Brain Calmodulins. The interaction of the isolated compounds with both spinach and brain calmodulins (Sigma) was performed using nondenaturing homogeneous electrophoresis (SDS−PAGE). SDS−PAGE was performed according to previously described procedures using 15% polyacrylamide gels.[4−6] The interaction of the phytotoxins with both calmodulins was determined by observing the difference in electrophoretic mobility in two different conditions, in the presence of Ca^{2+} and in the presence of EGTA. The experimental conditions are described briefly in the legend of Figure 1. In each case the electrophoresis run was done in triplicate. Quercetin was used as the positive control.

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References and Notes

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