Phytotoxic Compounds from *Prionosciadium watsoni*[1]

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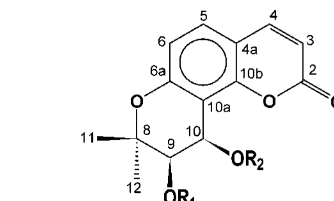
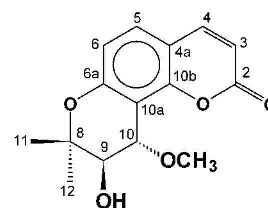
Bioassay-guided fractionation of a phytotoxic extract of *Prionosciadium watsoni* led to the isolation of three new pyranocoumarins and two pyranochromones. The new compounds were characterized as propionic acid (9*R*,10*R*)-9-acetoxy-8,8-dimethyl-9,10-dihydro-2*H*,8*H*-benzo[1,2-*b*:3,4-*b*′]dipyran-2-one-10-yl ester (**1**), isobutyric acid (9*R*,10*R*)-9-hydroxy-8,8-dimethyl-9,10-dihydro-2*H*,8*H*-benzo[1,2-*b*:3,4-*b*′]-dipyran-2-one-10-yl ester (**2**), isobutyric acid (9*R*)-8,8-dimethyl-9,10-dihydro-2*H*,8*H*-benzo[1,2-*b*:3,4-*b*′]dipyran-2-one-9-yl ester (**10**), 2-methylbut-(2*Z*)-enoic acid (3*R*)-5-methoxy-3,4-dihydro-2,2,8-trimethyl-6-oxo-2*H*,6*H*-benzo[1,2-*b*:5,4-*b*′]dipyran-3-yl ester (**11**), and isobutyric acid (3*R*)- 5-methoxy-3,4-dihydro-2,2,8-trimethyl-6-oxo-2*H*,6*H*-benzo[1,2-*b*:5,4-*b*′]dipyran-3-yl ester (**12**) by spectroscopic and chemical methods. The stereochemistry at the stereogenic centers was established by applying the Mosher ester methodology. The structures of **1** and **2** were corroborated by single-crystal X-ray diffraction studies. The phytotoxic activity of the isolated compounds was assessed on *Amaranthus hypochondriacus, Echinochloa crus-galli*, and *Lemna pausicostata.* The phytotoxins also modified the electrophoretic mobility of calmodulin from both bovine-brain and spinach.

Prionosciadium watsoni Coulter & Rose ex S.Watson (Umbelliferae), commonly known as "flor de ocotillo" and "hierba del oso", is a medicinal plant found in moist habitats of the pine-oak forest from Chihuahua to Hidalgo, Mexico. A tea prepared from the roots of this species is drunk to alleviate grastrointestinal pains, while the fruits are employed to treat diabetes, fever, and other diseases.[2] During the course of our search for potential herbicidal agents from Mexican plants, *P. watsoni* was selected for activity-guided fractionation on the basis of its significant phytotoxicity against *Amaranthus hypochondriacus* L., *Echinochloa crus-galli* (L.) Beuv., and *Lemna pausicostata* L. (duckweed). We report herein the isolation and structure elucidation of the major phytotoxins from *P. watsoni*.



	R_1	R_2		R_1	R_2
1	13, 14, O	15, 16, 17, O	**8**	**H**	**H**
2	**H**	13, 14, 15, 16, O	**1a**	**H**	**CH_3**
4	O	**H**	**1c**	**(*S*)-MTPA**	**CH_3**
6	O	O	**1d**	**(*R*)-MTPA**	**CH_3**
7	O	O			



1b

Results and Discussion

A CH_2Cl_2−MeOH (1:1) extract of the aerial parts of *P. watsoni* provoked notable inhibition of radicle elongation of seedlings of *A. hypochondriacus* and *E. crus-galli* (Table 1) when tested by the Petri dish germination and radicle elongation bioassay.[3] In addition, after 72 h in duckweed culture[4] the extract caused growth inhibition, chlorophyll reduction, and electrolytic leakage (Table 2). Bioassay-guided fractionation of the active extract, using the Petri dish test to monitor phytotoxicity, led to the isolation of phytotoxins **1**−**12**. Compounds **1**, **2**, **10**, **11**, and **12** are novel natural products, and their structures were determined by means of spectroscopic, spectrometric, and chemical methods as propionic acid (9*R*,10*R*)-9-acetoxy-8,8-dimethyl-9,10-dihydro-2*H*,8*H*-benzo[1,2-*b*:3,4-*b*′]dipyran-2-one-10-yl ester (**1**), isobutyric acid (9*R*,10*R*)-9-hydroxy-8,8-dimethyl-9,10-dihydro-2*H*,8*H*-benzo[1,2-*b*:3,4-*b*′]dipyran-2-one-10-yl ester (**2**), isobutyric acid (9*R*)-8,8-dimethyl-9,10-dihydro-2*H*,8*H*-benzo[1,2-*b*:3,4-*b*′]dipyran-2-one-9-yl ester (**10**), 2-methylbut-(2*Z*)-enoic acid (3*R*)-5-methoxy-3,4-dihydro-2,2,8-trimethyl-6-oxo-2*H*,6*H*-benzo[1,2-*b*:5,4-*b*′]dipyran-3-yl ester (**11**), and isobutyric acid (3*R*)-5-methoxy-3,4-dihydro-2,2,8-trimethyl-6-oxo-2*H*,6*H*-benzo[1,2-*b*:5,4-*b*′]dipyran-3-yl ester (**12**). In addition, the known compounds quianhucoumarin A (**3**),[5] 2-methylbut-(2*Z*)-enoic acid

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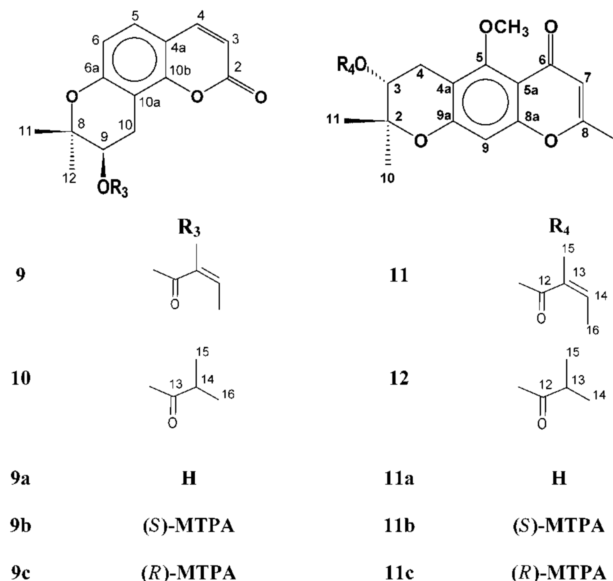
Table 1. Phytogrowth-Inhibitory Activity of the CH_2Cl_2−MeOH (1:1) Extract, Isolated Compounds, and Chemical Derivatives from *P. watsoni* on Radicle Elongation (IC_{50}, M) of *A. hypochondriacus* and *E. crus-galli*

treatment	*A. hypochondriacus*	*E. crus-galli*
extract[a]	0.68	2.12
1	3.96×10^{-4}	4.39×10^{-4}
2	1.89×10^{-4}	5.95×10^{-4}
4	2.62×10^{-4}	1.55×10^{-4}
6	4.02×10^{-4}	1.62×10^{-4}
7	ND[c]	ND[c]
8	3.61×10^{4}	4.39×10^{-4}
9	2.60×10^{-4}	8.63×10^{-4}
10	2.37×10^{-4}	1.69×10^{-4}
11	1.43×10^{-4}	2.64×10^{-5}
12	7.41×10^{-5}	1.48×10^{-5}
1a	3.25×10^{-4}	6.19×10^{-6}
1b	1.08×10^{-4}	8.08×10^{-4}
9a	3.44×10^{-4}	2.96×10^{-4}
11a	3.83×10^{-4}	4.61×10^{-4}
2,4-D[b]	1.80×10^{-4}	$>2.20 \times 10^{-4}$

[a] Expressed in μg mL^{-1}. [b] Positive control (2,4-dichlorophenoxyacetic acid). [c] Not determined because of the paucity of material.

(9*R*,10*R*)-10-hydroxy-8,8-dimethyl-9,10-dihydro-2*H*,8*H*-benzo[1,2-*b*:3,4-*b*′]dipyran-2-one-9-yl ester (**4**),[6] 2-methylbut-(2*E*)-enoic acid (9*R*,10*R*)-10-hydroxy-8,8-dimethyl-9,10-dihydro-2*H*,8*H*-benzo[1,2-*b*:3,4-*b*′]dipyran-2-one-9-yl ester (**5**),[7] seravshanin (**6**),[7] quianhucoumarin D (**7**),[5] (+)-*cis*-khellactone (**8**),[8,9] and jatamansin (**9**)[10] were obtained and identified by comparison of their published spectroscopic data.



Compounds **1** and **2** were isolated as colorless plates and needles, respectively. The molecular formulas were deduced as $C_{19}H_{20}O_7$ and $C_{18}H_{20}O_6$ by elemental analysis and ^{13}C NMR. Detailed analysis of the NMR (Table 3) spectra indicated that both compounds were *cis*-khellactone esters.[8,9] Compared to *cis*-khellactone[8,9] the NMR spectra of **1** showed additional resonances for one acetate and one propionate. Compound **2** differed from *cis*-khellactone in the presence of an isobutyroyl moiety. In the case of compound **1** the mass spectral fragmentation pattern suggested the position of the acetyl and propionyl units at C-9 and C-10, respectively, since the highest mass ion observed in the EIMS was at *m*/*z* 300 and corresponded to the loss of acetic acid from the molecular ion. In this regard, it is well known that khellactone diesters undergo a preferential loss of the ester moiety attached to C-9.[11,12] The isobutyrate residue in compound **2** was located at C-10 considering the chemical shift values for C-9/H-9 and C-10/H-10 as well as the C-9/H-11 and H-12 correlations observed in the HMBC spectrum. The absolute configuration at C-9 and C-10 was established by applying the Mosher ester methodology[13] on (+)-*cis*-methylkhellactone (**1a**). The latter compound was obtained by acid hydrolysis (HCl/MeOH) of **1** and **2**. Analysis of the Δ_{S-R} data (Table 4) for (+)-*cis*-methylkhellactone-*(S)*-MTPA (**2a**) and (+)-*cis*-methylkhellactone-*(R)*-MTPA (**2b**) derivatives showed that the absolute stereochemistry at C-9 was *R*. Thereafter, the absolute configuration at C-10 was automatically assigned as *R* because of the *cis* relationship between H-9 and H-10.

Table 2. Phytotoxicity of the Extract and Compounds **9** and **10** in *Lemna pausicostata* Cultures[a]

sample	concn	conductivity leakage (μΩ/cm)	growth inhibition (%)	chlorophyll reduction (%)
extract	25 μg mL^{-1}	−57.00 ± 14.8	3.01 ± 2.9	0.00 ± 12.7
	50 μg mL^{-1}	91.50 ± 39.9	7.79 ± 3.8	0.00 ± 0.0
	100μg mL^{-1}	262.50 ± 12.1	100.00 ± 0.0	36.74 ± 11.7
	200 μg mL^{-1}	262.50 ± 24.7	100.00 ± 0.0	91.51 ± 6.5
9	12 μM	−77.00 ± 42.4	15.33 ± 11.4	5.94 ± 4.9
	25 μM	−30.50 ± 31.1	37.51 ± 14.2	4.37 ± 4.3
	50 μM	59.50 ± 7.1	47.59 ± 5.7	12.82 ± 10.8
	100 μM	100.00 ± 9.2	100.00 ± 0.0	40.32 ± 3.3
10	25 μM	124.20 ± 21.1	19.59 ± 8.3	20.00 ± 16.8
	50 μM	100.00 ± 1.4	23.52 ± 13.8	54.30 ± 2.1
	100 μM	86.00 ± 2.1	58.81 ± 8.3	84.92 ± 4.0
	200 μM	260.00 ± 13.4	100.00 ± 0.0	87.38 ± 0.0

[a] Results are the means of three replicates ± standard deviation. The compounds exhibited significantly (Student's unpaired *t*-test) higher phytotoxicity than the control (culture medium; conductivity leakage, 0.00 ± 26.2 μΩ/cm; growth inhibition, 0.00 ± 2.7%; chlorophyll reduction, 0.00 ± 4.2%).

The structures of the novel coumarins **1** and **2** and that of the known compound **7** were confirmed by X-ray analysis (Figures 1−3). In all cases, the crystal structures clearly show the *syn* disposition of the substituents at C-9 and C-10. The pyranocoumarin skeleton of the molecules is essentially planar. The pyran ring adopts a half-chair conformation with C-8 and C-9 on opposite sides of the ring plane. In addition, both carbonyl oxygen atoms point toward the same direction and the ester group attached to C-10 displays some orientational disorder. Crystal packing of compounds **1** and **7** is governed by van der Waal forces, which are responsible for the crystal cohesion. For compound **2** the intramolecular hydrogen bonds between the hydroxy group (O-3) at C-9 and the coumarin oxygen atom (O-2) arrange the molecules in tetramers.

Compound **10** has a molecular formula of $C_{18}H_{20}O_5$ as inferred by EIMS, ^{13}C NMR, and elemental analysis. The ^1H NMR spectrum was similar to that of jatamansin (**9**)[10] and other lomatin analogues.[10] The most obvious differences between the NMR spectra of compounds **9** and **10** resulted from the presence of the signals for an isobutyroyl moiety in **10** instead of the resonances for the angeloyl unit in **9**. The absolute configuration at C-9 was also accomplished by applying the Mosher ester metodology[13] on (+)-lomatin (**9a**), obtained by prolonged treatment of **10** with boiling methanolic KOH. Analysis of the Δ_{S-R} data (Table 4) for the (+)-lomatin-*(S)*-MTPA (**9b**) and (+)-lomatin-*(R)*-MTPA (**9c**) derivatives showed that the absolute stereochemistry at C-9 was *R*. In accordance with the above discussion **10** was characterized as isobutyric acid (9*R*)-8,8-dimethyl-9,10-dihydro-2*H*,8*H*-benzo[1,2-*b*:3,4-*b*′]-dipyran-2-one-9-yl ester.

Table 3. NMR Data for Compounds **1**, **2**, and **10**[a]

position	**1** δ_H (m, J in Hz)	**1** δ_C[b]	**2** δ_H (m, J in Hz)	**2** δ_C[b]	**10** δ_H (m, J in Hz)	**10** δ_C[b]
2		159.8		159.9		161.2
3	6.23 (d, 9.5)	113.2	6.23 (d, 9.6)	113.0	6.23 (d, 9.50)	112.6
4	7.59 (d, 9.5)	143.2	7.59 (d, 9.6)	143.3	7.63 (d, 9.50)	143.8
4a		112.6		112.5		112.2
5	7.35 (d, 8.5)	129.1	7.36 (d, 8.5)	129.3	7.26 (d, 8.8)	126.7
6	6.80 (d, 8.5)	114.4	6.81 (d, 8.5)	114.5	6.79 (d, 8.8)	114.3
6a		156.7		156.9		153.4
8		77.4		78.6		76.6
9	5.31 (d, 5.0)	70.1	4.05 (d, 4.8)	71.7	5.11 (t, 5.5)	69.2
10	6.55 (d, 5.0)	60.8	6.38 (d, 4.8)	63.4	3.21 (dd, 5.5, 17.6)	23.0
10′					2.92 (dd, 5.5, 17.6)	
10a		107.0		107.6		107.2
10b		154.0		154.2		156.3
11	1.44 (s)	22.7	1.48 (s)	22.3	1.36 (s)	22.4
12	1.41 (s)	24.8	1.42 (s)	20.7	1.38 (s)	24.8
−OH			2.37 (brs)			
13		170.0		178.5		176.2
14	2.09 (s)	20.7	2.68 (hept, 6.5)	34.3	2.55 (hept, 7.00)	34.0
15		173.3	1.26 (d, 6.5)	18.8	1.17 (dd, 7.00)	18.8
16	2.43 (dq, 7.5,16.0)	27.5	1.23 (d, 6.5)	18.9	1.14 (dd, 7.00)	19.0
16′	2.38 (dq, 7.5,16.0)					
17	1.20 (dd, 7.5)	9.1				

[a] Spectra recorded in $CDCl_3$ (^1H, 500 MHz and ^{13}C, 125 MHz); δ in ppm. [b] Assigned by HMQC, HMBC, and DEPT.

Table 4. Partial ^1H NMR Data of the (S)- and (R)-Mosher Esters of **1a** and **9a**[a]

proton	**1a** (S)-MTPA	**1a** (R)-MTPA	**1a** $\Delta\delta_{S-R}$	**1a** carbinol confign.	**9a** (S)-MTPA	**9a** (R)-MTPA	**9a** $\Delta\delta_{S-R}$	**9a** carbinol confign.
9	5.306	5.346		R	5.246	5.245		R
10	4.963	4.882	+0.081		3.311	3.266	+0.045	
					3.065	2.981	+0.84	
11[b]	1.401	1.494	−0.093		1.323	1.378	−0.055	
12[b]	1.347	1.399	−0.052		1.273	1.364	−0.091	

[a] Spectra recorded in $CDCl_3$ (300 MHz). [b] Interchangeable signals.

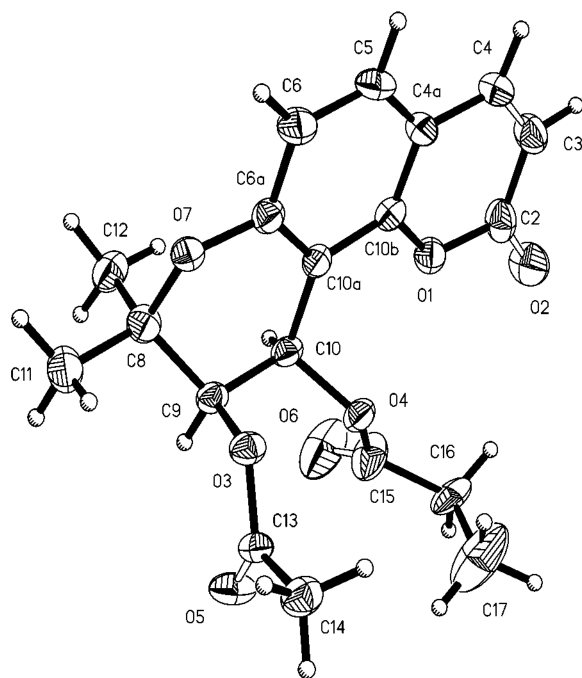


Figure 1. X-ray crystal structure for **1**.

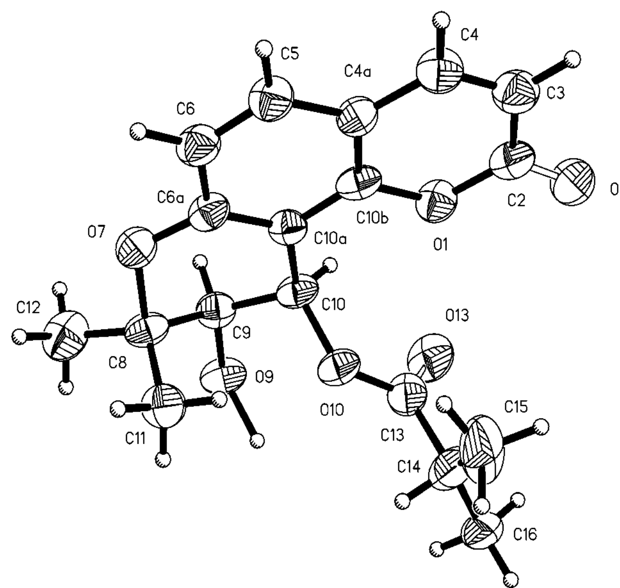


Figure 2. X-ray crystal structure for **2**.

The molecular formulas of compounds **11** and **12** were determined as $C_{21}H_{24}O_6$ and $C_{20}H_{24}O_6$, respectively, by MS, NMR, and elemental analysis. Their NMR (Table 5), UV, and IR spectra indicated that these compounds are linear pyranochromones related to (−)-hamaudol,[14] a linear pyranochromone possessing a 3(S)-hydroxy functionality. The NMR spectra (Table 5) of **11** and **12** showed signals due to the presence of two carbonyl groups, a methoxy functionality, five methyl groups, and two methines in the aromatic region. The main differences between the NMR spectra (Table 5) of compounds **11** and **12** resulted in the signals due to the ester moiety at C-3. In **12** these signals were consistent with the presence of an isobutyrate, while in **11** with an angelate. Cursory inspection of the HMBC spectra of **11** and **12**, which displays the correlations summarized

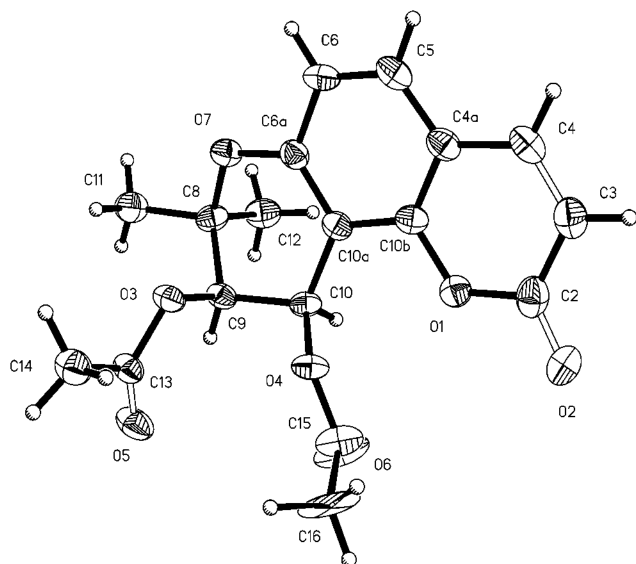


Figure 3. X-ray crystal structure for **7**.

in Table 5, confirmed the pyranochromone skeleton as well as the position of the methoxy, methyl, and ester groups in both molecules. Upon alkaline hydrolysis (KOH/MeOH), **11** and **12** yielded (+)-5-methoxyhamaudol (**11a**), which is also a new analogue of (−)-hamaudol. The NMR spectra of **11a** (see Experimental Section) differ from those of hamaudol[14] in the presence of the signal for the methoxyl group at C-5. The sign of the optical rotation of **11a** is opposite that of hamaudol, suggesting that the stereochemistry at C-3 of **11** and **12** is the opposite that of (−)-hamaudol. The application of the advanced Mosher ester methodology[13] on **11a** corroborated this proposal since analysis of the Δ_{S-R} data (Table 6) for the (+)-5-methoxyhamaudol-*(S)*-MTPA (**11b**) and (+)-5-methoxyhamaudol-*(R)*-MTPA (**11c**) derivatives clearly indicated that the absolute stereochemistry at C-3 was *R*.

The phytotoxic activity of the isolates and chemical derivatives **1a**, **1b**, **9a**, and **11a** was determined on germination and radicle elongation of *A. hypochondriacus* and *E. crus-galli* seedlings using 2,4-D (2,4-dichlorophenoxyacetic acid) as the positive control. Table 1 summarizes the phytotoxic effect (expresed as IC_{50} values) of the tested compounds. In general, the compounds inhibited radicle growth in a concentration-dependent manner. Compounds **11**, **12**, and **1a** were the most active and showed high selectivity against *E. crus-galli* seeds. Of all the isolates, only **9** and **10** showed significant phytotoxicity on duckweed at concentrations of 100 and 200 μM (Table 2). These compounds caused growth inhibition, chlorophyll reduction, and electrolytic leakage. Apparently, during the course of this study it was not possible to isolate the active compound responsible for the marked phytotoxicity on duckweed because the extract was more active than compounds **9** and **10**. On the other hand, it is possible that the compounds present in the extract exerted a synergistic effect on duckweed.

Continuing with our search of potential herbicide agents that could interact with calmodulin (CaM), the phytotoxic compounds were further evaluated for their ability to bind spinach and bovine brain CaM.[15,16] CaM is an intracellular receptor for Ca^{2+} in eukaryotes which modulates enzymatic response to calcium. This protein plays a leading role in plant signal transduction during germination and plant growth, modulating the activity of several important plant enzymes such as NAD-kinase, gluatamate decarboxilase, and Ca^{2+}-ATPase. Therefore, CaM and/or the enzymes it regulates could be important targets in vivo for the phytotoxic action of many phytotoxins.[17] In this context, it has been previously described that some phytotoxins such as ophiobolin A interact with CaM, altering its enzyme activator properties.[18] The interaction of ophiobolin A with CaM has been demonstrated by electrophoresis, by fluorescence experiment, and by evaluating its capability of inhibiting the activation of cAMP-phosphodiesterase.[18] Concerning the first experiment, it is well known that the interaction of any compound with Ca^{2+}-CaM complex alters its conformation and changes its electrophoretic mobility; however, it does not reveal if the tested compound modifies the enzyme activator properties of CaM.[15,18] Figure 4 shows that all compounds tested (0.033 μg mL^{-1}) modify the electrophoretic mobility of both proteins. Thus, these compounds interact with calmodulin and, as ophiobolin A, might exert their phytotoxic action by interfering with calmodulin-regulated processes in vivo.

In conclusion, the results of the present investigation revealed that *P. watsoni* contains phytotoxins that interact with Ca^{2+}-calmodulin. These compounds might be useful leads for the development of new herbicide agents. Further work is in progress in order to evaluate if this interaction affects calmodulin function.

Experimental Section

General Experimental Procedures. Melting points were determined on a Fisher-Johns apparatus and are uncorrected. IR spectra were measured on a Perkin-Elmer 599 spectrometer. UV spectra were obtained on a Shimadzu 160 UV spectrometer in MeOH solution. Optical rotations were taken on a JASCO DIP-360 polarimeter. NMR spectra, including COSY spectra, NOESY, HMQC, and HMBC experiments were obtained on a Varian UNITY PLUS 500 spectrometer in $CDCl_3$ or MeOD at either 300 or 500 MHz (^{1}H), or 75 or 125 MHz (^{13}C), or on a Bruker DMX500 at 500 MHz (^{1}H) or 125 MHz (^{13}C) using tetramethylsilane (TMS) as an internal standard. EIMS data were recorded on a JEOL JMS-AX505HA mass spectrometer, at an ionization energy of 70 eV. HPLC was carried out with a Waters UV photodiode array detector (900) set at 190−350 nm, using a preparative silica gel column (μ-porasil 10 Å, 100 mm i.d. × 300 mm). Control of the equipment, data acquisition, processing, and management of chromatographic information were performed by the Millenium 2001 software program (Waters). Open column chromatography: Si gel 60 (70−230 mesh, Merck). Analytical and preparative TLC: Si gel 60 F_{254} (Merck).

Plant Material. The aerial parts of *P. watsoni* were collected on February 12, 1999, at San Luis Potosí, Mexico (Sierra Alvarez, Municipio Villa de Zaragoza), and identified by Dr. Robert Bye, Instituto de Biología UNAM. A voucher specimen (R. BYE et al. 26911) is preserved at the Ethnobotanical Collection of the National Herbarium (MEXU), Instituto de Biología, UNAM.

Extraction and Isolation of 1−12. The air-dried and ground aerial parts of *P. watsoni* (1.8 Kg) were extracted with CH_2Cl_2−MeOH (1:1). The resulting extract (345.2 g) was subjected to open Si gel (1200 g) column cromatography and eluted with a gradient of hexane−EtOAc (1:0→ 0:1) and EtOAc−MeOH (9:1→1:1) mixtures. Altogether, 49 fractions were collected and combined according to their TLC patterns to yield eight primary fractions (F_1−F_8). Bioactivities in the Petri dish germination and radicle elongation bioassay showed three active pools: F_2 (160.4 g, IC_{50} = 53.3 μg mL^{-1} for *A. hypochondriacus*; IC_{50} = 1114.5 μg mL^{-1} for *E. crus-galli*), F_4 (144 g, IC_{50} = 241.6 μg mL^{-1} for *A. hypochondriacus*; IC_{50} = 56.1 μg mL^{-1} for *E. crus-galli*), and F_5 (12.7 g, IC_{50} = 56.2 μg mL^{-1} for *A. hypochondriacus*; IC_{50} = 174.7 μg mL^{-1} for *E. crus-galli*). From the active fraction F_4, eluted with hexane−EtOAc, 7:3, precipitated a mixture of **1**, **6**, and **7** (35 g). Part of this

Table 5. NMR Data for Pyranochromones **11** and **12**[a]

	11			**12**		
position	δ_H (m, *J* in Hz)	δ_C[b]	HMBC (C → H)	δ_H (m, *J* in Hz)	δ_C[b]	HMBC (C → H)
2		81.9	4,10,11		81.6	4,10,11
3	5.03 (dd, 7.0)	89.3	4,11,10	5.01 (dd, 7.0)	89.0	4,10,11
4α	3.33 (dd, 7.0, 9.5)	27.7		3.30 (dd, 7.0, 9.6)	27.6	
4β	3.27 (dd, 7.0, 9.5)			3.23 (dd, 7.0, 9.6)		
4a		116.7	4,9		116.7	4,9
5		155.8	$-OCH_3$,4		155.7	$-OCH_3$,4
5a		111.9	9		111.5	9
6		177.1			177.1	
7	5.99 (q, 0.5)	111.4	$-CH_3$	5.99 (q, 0.75)	111.4	9,$-CH_3$
8		163.3	7,$-CH_3$		163.3	7,$-CH_3$
8a		159.9	9,$-CH_3$		159.9	9,$-CH_3$
9	6.52 (s)	93.7		6.53 (s)	93.7	
9a		164.4	4,9		164.4	4,9
10	1.62 (s)	21.4		1.56 (s)	21.1	
11	1.61 (s)	22.1		1.53 (s)	22.0	
12		167.1	15		167.2	13,14,15
13		128.5	15,16	2.41 (hept, 7.0)	34.9	14,15
14	5.98 (ddq, 1.5, 7.5, 14.5)	137.7	15,16	1.06 (d, 7.0)	18.8	13
15	1.89 (dq, 1.5, 7.5)	15.6	16	1.05 (d, 7.0)	18.8	13
16	1.68 (q, 1.5, 7.0)	20.6	15			
$-OCH_3$	5.93 (s)	61.1		3.94 (s)	61.1	
$-CH_3$	2.27 (d, 0.5)	19.7	7	2.28 (d, 0.75)	19.8	7

[a] Spectra recorded in $CDCl_3$ (^1H, 500 MHz and ^{13}C, 125 MHz); δ in ppm. [b] Assigned by HMQC, HMBC, and DEPT.

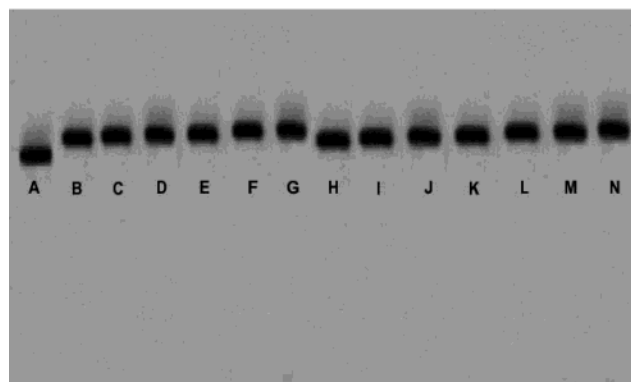
Table 6. Partial ^1H NMR Data of the (*S*)- and (*R*)-Mosher Esters of **11a**[a]

proton	(*S*)-MTPA	(*R*)-MTPA	$\Delta\delta_{S-R}$	carbinol confign.
3	4.867	4.095		*R*
4α	3.079	3.067	+0.012	
4β	3.253	3.247	+0.006	
10	1.664	1.734	−0.070	
11	1.661	1.725	−0.060	

[a] Spectra recorded in $CDCl_3$ (300 MHz).

mixture (200 mg) was resolved by extensive preparative TLC (hexane−EtOAc, 3:1) to yield **1** (60 mg), **6** (130 mg), and **7** (1 mg). The mother liquors from phytotoxic fraction F_4 were rechromatographed on a Si gel (734 g) column eluting with a gradient of hexane−EtOAc (1:0 → 0:1) to yield 19 secondary fractions (F_I−F_{XIX}). According to the Petri dish germination and radicle elongation bioassay, the phytotoxic activity was concentrated in secondary fractions F_{4VI} (6.07 g) and $F4_{IX}$ (8.6 g). Secondary fraction F_{4VI}, eluted with hexane−EtOAc, 9:1, was chromatographed on a Si gel (81 g) column eluting with a gradient of hexane−EtOAc (1:0 → 0:1) to afford 10 tertiary fractions. The phytotoxic activity was concentrated in fraction F_{4VI-6} (4.9 g). Extensive TLC (hexane−EtOAc, 3:1) of active fraction F_{4VI-6} (200 mg), eluted with hexane−EtOAc, 9:1, yielded **9** (50 mg) and **10** (12 mg). Secondary active fraction F_{4IX}, eluted with hexane−EtOAc, 8:2, was further chromatographed on another Si gel (151.0 g) column eluting with a gradient of hexane−EtOAc (1:0 → 0:1) and EtOAc−MeOH (1:1) to yield nine tertiary fractions (F_{4-IX-1}−F_{4-IX-9}). The phytotoxic activity was concentrated in fraction F_{4-IX-5} (10.89 g), eluted with hexane−EtOAc, 7:3. The latter fraction was rechromatographed on a Si gel (217.8 g) column eluting with a gradient of CH_2Cl_2−MeOH (1:0 → 1:1) to give seven quaternary fractions ($F_{4-IX-5-I}$−$F_{4-IX-5-VII}$). The phytotoxic activity was concentrated in fraction $F_{4-IX-5-V}$ (4.9 g), eluted with CH_2Cl_2−MeOH, 99.95:0.05. Extensive HPLC purification of the active quaternary fraction $F_{4-IX-5-V}$ (100 mg) on a normal-phase Si gel column [10 mL min^{-1}, Hex−*i*-PrOH−MeOH (95:2.5:2.5), λ 230 nm] yielded **2** (15 mg), **3** (5 mg), **4** (5 mg), **5** (5 mg), and additional amounts of **1** (20 mg); retention times: 18.6, 19.1, 26.3, 27.4, and 29.5 min, respectively. Active fraction F_5, eluted with hexane−EtOAc, 6:4, was further resolved by column chromatograpy on Si gel (182 g) eluting with hexane−EtOAc (1:0 → 0:1) and EtOAc−MeOH (9:1 → 1:1) mixtures. This process yielded 23 secondary fractions (F_{5-I}−$F_{5-XXIII}$), and the Petri dish bioassay indicated that the phytotoxic activity was in secondary fractions F_{5-XII} (607.4 mg) and F_{5-XIII} (1.5 g). Further purification of active fracion F_{5-XII} (eluted with hexane−EtOAc, 6:4) by HPLC [10 mL min^{-1}, Hex−*i*-PrOH−MeOH (95:2.5:2.5), λ 230 nm] yielded **8** (20 mg),

a



b

Figure 4. SDS-PAGE of bovine (a) and spinach (c) calmodulins after treatment with isolates and chemical derivatives. Electrophoresis of 2 μg samples of calmodulins in the presence of 1 mM $CaCl_2$. Pretreatments of the calmodulin samples, for 1.5 h at 30 °C in the presence of $CaCl_2$: A, no additions; B, 0.033 μg/mL (2*S*,3*S*)-5-hydroxy-6,8,10-trimethoxy-2,3-dimethyl-4*H*-dihydronaphtho[2,3-*b*]pyran-4-one; C, **1**; D, **2**; E, **4**, F, **6**; G, **9**; H, **10**; I, **11**; J, **12**; K, **1a**; L, **1b**; M, **9a**; N, **11a**. In all cases 0.033 μg/mL of treatment (**1**−**11a**) was applied.

retention time 25.2 min. Extensive HPLC purification of the active secondary fraction F_{5-XIII} (200 g), eluted with hexane−EtOAc, 6:4, on a normal-phase Si gel column [10 mL min^{-1}, Hex−*i*-PrOH−MeOH (90:5.0:5.0), λ 230 nm] yielded **11** (60 mg) and **12** (10 mg); retention times: 20.2 and 20.9 min, respectively.

Propionic acid (9*R*,10*R*)-9-acetoxy-8,8-dimethyl-9,10-dihydro-2*H*,8*H*-benzo[1,2-*b*:3,4-*b*′]dipyran-2-one-10-yl ester (1): colorless plates, mp 208 °C; $[\alpha]^{20}$ +18 (*c* 1 mg mL^{-1} $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 321 (4.23) nm; IR ν_{max} (KBr) 3097, 2980, 1746, 1605, 1225, 1024 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz), see Table 3; EIMS *m*/*z* 300 [M$^+$ − AcOH (29)], 285 (8), 261 (6), 244 (30), 229 (100), 57 (21), 43 (15). Anal. Calcd for $C_{19}H_{20}O_7$: C 63.33, H 5.59. Found: C 63.39, H 5.53.

Isobutyric acid (9*R*,10*R*)-9-hydroxy-8,8-dimethyl-9,10-dihydro-2*H*,8*H*-benzo[1,2-*b*:3,4-*b*′]dipyran-2-one-10-yl ester (2): colorless needles, mp 134 °C; $[\alpha]^{20}$ +151 (*c* 1 mg mL^{-1} $CHCl_3$); UV (MeOH)) λ_{max} (log ϵ) 323 (4.27) nm; IR ν_{max} (KBr) 3475, 2977, 2926, 1730, 1607, 1234, 1108 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz), see Table 3; EIMS *m*/*z* 332 [M$^+$(16)], 314 [M$^+$ − H_2O(5)], 299 (17), 261 (18), 244 (13), 229 (100), 215 (9), 191 (27), 175 (11), 71 (24), 43 (29). Anal. Calcd for $C_{18}H_{20}O_6$: C 65.05, H 6.07. Found: C 65.00, H 6.09.

Isobutyric acid (9*R*)-8,8-dimethyl-9,10-dihydro-2*H*,8*H*-benzo[1,2-*b*:3,4-*b*′]dipyran-2-one-9-yl ester (10): vitreous colorless solid, mp 108−110 °C; $[\alpha]^{20}$ +20 (*c* 1 mg mL^{-1} $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 324 (3.9) nm; IR ν_{max} (film) 2979, 2944, 1732, 1606, 1144, 1113; ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz), see Table 3 EIMS *m*/*z* 316 [M$^+$(19)], 246 (3), 228 (4), 213 (100), 83 (7), 71 (17), 55 (6), 43 (29). Anal. Calcd for $C_{18}H_{20}O_5$: C 68.34, H 6.37. Found: C 68.38, H 6.30.

2-Methylbut-(2Z)-enoic acid (3*R*)-5-methoxy-3,4-dihydro-2,2,8-trimethyl-6-oxo-2*H*,6*H*-benzo[1,2-*b*:5,4-*b*′]dipyran-3-yl ester (11): yellow oil; $[\alpha]^{20}$ +340 (*c* 1 mg mL^{-1} $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 214 (4.50), 285 (4.12) nm; IR ν_{max} (film) 2981, 2987, 1714, 1660, 1621, 1470, 1238, 1141 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz), see Table 5; EIMS *m*/*z* 372 [M$^+$(25)], 289 (2), 272 (23), 257 (100), 83 (30). Anal. Calcd for $C_{21}H_{24}O_6$: C 67.73, H 6.50. Found: C 67.78, H 6.49.

Isobutyric acid-(3*R*)- 5-methoxy-3,4-dihydro-2,2,8-trimethyl-6-oxo-2*H*,6*H*-benzo[1,2-*b*:5,4-*b*′]dipyran-3-yl ester (12): vitreous solid, mp 140−141 °C; $[\alpha]^{20}$ +560 (*c* 1 mg mL^{-1} $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 213 (4.6), 284 (4.10) nm; IR ν_{max} (KBr) 2972, 2926, 1731, 1655, 1617, 1468, 1378, 1143 cm^{-1}. ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz), see Table 5; EIMS *m*/*z* 360 [M$^+$(29)], 273 (27), 257 (100), 231 (55), 229 (51), 71 (8), 43 (13). Anal. Calcd for $C_{20}H_{24}O_6$: C 66.65, H 6.71. Found: C 66.62, H 6.75.

Preparation of (+)-*cis*-Methylkellactone (1a) and (−)-*trans*-Methylkhellactone (1b). A solution of **1** (50 mg) in 3 mL of MeOH was mixed with 3.5 mL of 1 N methanolic HCl and refluxed for 22 h. The reaction mixture was allowed to stand for 45 min and adjusted to pH 8 with a $NaHCO_3$ (10% aqueous) solution, concentrated in vacuo to remove the MeOH, and extracted with CH_2Cl_2. The CH_2Cl_2 layers were combined, washed with water, and dried over Na_2SO_4. The reaction mixture was chromatographed on preparative TLC [hexane−EtOAc (7:3)] to yield **1a** (10 mg) and **1b** (15 mg). **1a:** mp 118−119 °C, $[\alpha]^{20}$ +690 (*c* 1 mg mL^1 $CHCl_3$); **1b:** mp 157−158 °C; $[\alpha]^{20}$ −336 (*c* 1 mg/mL $CHCl_3$). The spectroscopic and spectrometric data of **1a** and **1b**, including UV, IR, ^{1}HNMR, and ^{13}CNMR, were identical to those previously described.[19]

Preparation of (+)-Lomatin (9a). To a solution of **9** or **10** (10 mg each) in 2 mL of MeOH was added 2 mL of 1 N methanolic KOH, and the mixture was refluxed for 3.5 h. The reaction mixture was diluted with water, concentrated in vacuo to remove the organic solvent, acidified with 2 N sulfuric acid (2 mL), and extracted with EtOAc. The combined EtOAc layers were succesively washed with 10% aqueous $NaHCO_3$ solution and water and dried over Na_2SO_4. The resulting residues were purified by preparative TLC [hexane−EtOAc (1:1)] to give **9a** (5 mg), mp 184−185 °C; $[\alpha]^{20}$ +210 (*c* 1 mg mL^{-1} $CHCl_3$). The spectroscopic and spectrometric data, including UV, IR, and ^{1}HNMR, were the same as those previously described.[10]

Preparation of (+)-5-Methoxyhamaudol (11a). A solution of **11** (10 mg) or **12** (5 mg) in 3 mL of MeOH was treated as described for **9** and **10**. The reaction mixtures were purified by column chromatography on Si gel [2 g, EtOAc] to give **11a** (5 and 2 mg respectively), mp 137−138 °C; $[\alpha]^{20}$ +560 (*c* 1 mg mL^{-1} $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.02 (1H, q, J = 0.75 Hz, H-3), 6.61 (1H, s, H-6), 4.75 (1H, dd, J = 4.5, 8.24 Hz, H-3′), 3.34 (1H, dd, J = 4.25, 8.24 Hz, H-4′), 1.29 (3H, s, H-5′), 1.23 (3H, s, H-5′), 2.32 (1H, d, J = 0.75 Hz, CH_3), 3.92 (1H, s, $-OCH_3$); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 179.80 (C-2), 166.58 (C-4), 166.43 (C-7), 161.44 (C-5), 156.96 (C-8a), 118.47 (C-8), 111.54 (C-4a), 111.49 (C-3), 92.65 (C-2′), 72.25 (C-3′), 61.04 ($-OCH_3$), 28.72 (C-4′), 25.4 (C-5′), 25.31 (C-6′), 19.71 ($-CH_3$); EIMS *m*/*z* 290 [M$^+$(100)], 272 (18), 257 (23), 243 (13), 231 (88), 213 (87), 201 (57), 189 (32), 186 (24), 174 (11), 160 (8), 132 (8), 59 (39), 43 (9). Anal. Calcd for $C_{16}H_{18}O_5$: C 66.20, H 6.25. Found: C 66.25, H 6.24.

Mosher Esters of Compounds 1a, 9a, and 11a. 1a (3.0 mg), **9a** (3.0 mg), or **11a** (3.0 mg) was dissolved in CH_2Cl_2 (1 mL) and treated with (*S*)- or (*R*)-MTPA (18 mg), DCC (18 mg), and 4-DMAP (4 mg). The mixtures were stirred at room temperature (25 °C) for 3 h and then poured into ice−water. The resulting mixtures were then extracted with $CHCl_3$; the organic phases were successively washed with 5% aqueous HCl, saturated $NaHCO_3$, and water and then dried over Na_2SO_4 and filtered. Evaporation of the solvent under reduced pressure afforded residues which were purified by chromatography on Si gel [2 g, hexane−EtOAc (7:3)] for **1a** and **9a**. In the case of Mosher esters of compound **11a** the column was eluted with EtOAc to yield the corresponding (*S*)- and (*R*)-MTPA esters.

X-ray Crystallographic Analysis of Compounds 1, 2, and 7.[20] Molecular structures of compounds **1**, **2**, and **7** were analyzed by X-ray diffraction methods following very similar procedures. For each sample, crystals were mounted, in air, on glass fibers. Accurate cell parameters were determined by refinement from the setting of 25 reflections and diffraction intensities measured at 293 K using an $\omega-\theta$ scan method on a Siemens P4/PC diffractometer equipped with graphite-monochromated radiation. The intensities of three standard reflections, recorded every 100 collected reflections, showed no changes. All data sets were corrected for Lorentz−polarization effects, but no absorption corrections were applied. The structure of each compound was determined by direct methods (SIR92)[21] and refined by full-matrix least-squares methods using SHELXL97.[22] Hydrogen atoms attached to C atoms were set to ride on the parent C atoms, and for those bonded to O atoms their positional parameters were refined; an isotropic temperature factor 1.2 times the U_{eq} of the parent atom was used. The non-hydrogen atoms were refined with anisotropic thermal parameters.

Crystal Data for Compound 1: $C_{19}H_{20}O_7$, M_r = 360.35, colorless plates with dimensions 0.32 × 0.26 × 0.06 mm, orthorhombic. The space group is $P2_12_12_1$ with unit cell parameters (at 25 °C) a = 8.5950(4), b = 10.394(1), c = 20.015(1) Å, V = 1788.07(19) Å^3, Z = 4, F(000) = 752, d_{calcd} = 1.331 g cm^{-3}, μ/mm^{-1} = 0.861.

Crystal Data for Compound 2: $C_{18}H_{20}O_6$, M_r = 332.34, colorless needle with dimensions 0.60 × 0.12 × 0.10 mm, monoclinic. The space group is C_2 with unit cell parameters (at 25 °C) a = 29.101(3), b = 9.010(1), c = 14.373(1) Å, and V = 3535.2(6) Å^3, Z = 8, F(000) = 71408, d_{calcd} = 1.249 g cm^{-3}, μ/mm^{-1} = 0.782.

Crystal Data for Compound 7: $C_{18}H_{20}O_7$, M_r = 346.32, colorless prism with dimensions 0.60 × 0.32 × 0.10 mm, orthorombic. The space group is $P2_12_12_1$ with unit cell parameters (at 25 °C) a = 8.631(1), b = 10.403(2), c = 20.111(5) Å, and V = 1805.7(6) Å^3, Z = 4, F(000) = 728, d_{calcd} = 1.274 g cm^{-3}, μ/mm^{-1} = 0.099.

Bioassay Measuring Plant Growth Inhibition. The phytogrowth inhibitory activity on the extract and pure compounds on seedlings of *A. hypochondriacus* and *E. crus-galli* was evaluated using the Petri dish radicle elongation and

germination bioassay.[3] 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 1, 10, 100, and 100 μg mL^{-1}. The fractions and pure compounds were tested at 10, 50, 100, and 200 μg mL^{-1}. 2,4-D was used as the positive control. The bioassays were performed at 28 °C.

Duckweed Bioassay. The duckweed assay was carried out essentially as described in detail by Tanaka et al.[4] The extract at concentrations of 10, 100, and 1000 μg mL^{-1} and the pure compounds at concentrations of 25, 50, 100, and 200 μM in half-strength Hunter's medium[22] were added to 10 duckweed colonies of three fronds each. In the experiment 3 mL of media was used and the test was run for 3 days. Electrolyte leakage was determined by a conductivity meter at the beginning and during 72 h. Chlorophyll was determined based on fresh weight by first removing duckweed from bathing media, blotting with paper towels, and extracting with 5 mL of DMSO by soaking overnight in the dark. The total chlorophyll (a and b) was determined by ultraviolet spectroscopy according to Hiscox and Israelstam.[24] Growth inhibition was measured by determining duckweed fresh weight at the beginning and the end of the experiment.

Evaluation of the Interaction of Isolated Compounds and Derivatives with Spinach and Bovine Brain Calmodulins. The interaction of the isolated compounds and derivatives with both spinach and brain calmodulins (Sigma) was performed through denaturing homogeneous electrophoresis (SDS-PAGE). SDS-PAGE was performed according to previously described procedures using 15% polyacrylamide gels.[15,16] The interaction of the phytotoxins with both calmodulins was evaluated by observing the difference in the electrophoretic mobility in the presence of Ca^{2+}. Each electrophoretic run was done by trplicate, and the compound (2*S*,3*S*)-5-hydroxy-6,8,10-trimethoxy-2,3-dimethyl-4*H*-dihydronaphtho-[2,3-*b*]pyran-4-one[15,16] was used as the positive control.

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Supporting Information Available: Experimental crystallographic details, tables of atomic coordinates and equivalent isotropic displacement parameters, and hydrogen bond schemes for compounds **1**, **2**, and **7**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(1) This work represents part of the Ph.D. Thesis submitted to the graduate school of Chemistry UNAM, Mexico, by N.V.
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