Phytotoxins from *Hofmeisteria schaffneri*: Isolation and Synthesis of 2′-(2″-Hydroxy-4″-methylphenyl)-2′-oxoethyl Acetate[1]

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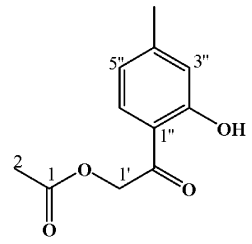
Activity-directed fractionation of a CH_2Cl_2−MeOH (1:1) extract of *Hofmeisteria schaffneri* led to the isolation of a new phytotoxin characterized as 2′-(2″-hydroxy-4″-methylphenyl)-2′-oxoethyl acetate and designated the trivial name of hofmeisterin (**1**). In addition, the known compounds β-carotene, euparin, and 3′,4′,4a′,9a′-tetrahydro-6,7′-dimethylspiro[benzofuran-3(2*H*),2′-pyrano[2,3-*b*]benzofuran]-2,4a′-diol (**2**) were obtained. The identification of the isolates was accomplished by spectroscopic methods. The structure of **1** was unequivocally confirmed by synthesis. The methyl derivative **1a** was also synthesized following the same strategy. Compounds **1** and **2** inhibited radicle growth of *Amaranthus hypochondriacus* (IC_{50} = 3.2×10^{-4} and 1.2×10^{-5} M, respectively) and significantly inhibited activation of the calmodulin (CaM)-dependent enzyme cAMP phosphodiesterase (PDE) with IC_{50} values of 4.4 and 4.22 μM, respectively.

As part of our systematic search of potential herbicidal agents from Mexican biodiversity,[2−4] we have selected *Hofmeisteria schaffneri* (A. Gray) R. M. King & H. Robinson (Asteraceae) for bioassay-guided fractionation on the basis of its significant phytotoxic activity against *Amaranthus hypochondriacus* and *Echinochloa crus-galli*. *H. schaffneri*, a rare perennial medicinal herb known as "ambar", grows naturally in the oak and pine-oak forested mountains of the central Mexican states of Jalisco and San Luis Potosi and is cultivated in home gardens in the valley of Mexico. The decoction of the fresh, leafy stems and flowers is used for treating skin wounds, fevers, and gastrointestinal ailments.[5] Herein, we describe the isolation, structure elucidation, and biological activity of the major phytotoxins from *H. shaffneri* including their effect on the regulatory protein calmodulin (CaM) as a possible target of phytotoxic action. The synthesis of 2′-(2″-hydroxy-4″-methylphenyl)-2′-oxoethyl acetate (**1**), a novel phytotoxin isolated during the course of the present investigation, is also reported.



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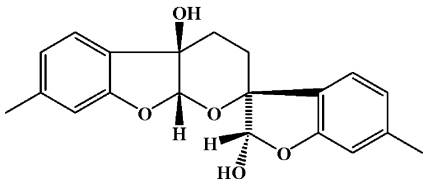

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A crude CH_2Cl_2−MeOH (1:1) extract prepared from the plant *H. schaffneri* showed an inhibitory effect on radicle growth of *Amaranthus hypochondriacus* (IC_{50} = 52.5 μg/mL) and was accordingly subjected to bioassay-guided fractionation. Extensive chromatography of the active extract resulted in the isolation of 2′-(2″-hydroxy-4″-methylphenyl)-2′-oxoethyl acetate (**1**), a novel phytotoxin that was given the trivial name of hofmeisterin. The known compounds euparin,[6] β-carotene,[7,8] and 3′,4′,4a′,9a′-tetrahydro-6,7′-dimethylspiro[benzofuran-3(2*H*),2′-pyrano[2,3-*b*]benzofuran]-2,4a′-diol (**2**)[9] were also isolated. The properties of the known compounds, including IR, ^{1}H NMR, and ^{13}C NMR data, were identical to those previously reported in the literature.

Compound **1** was assigned the molecular formula $C_{11}H_{12}O_4$ (HRMS). The IR spectrum showed bands for phenolic (3500 cm^{-1}), ester (1747 cm^{-1}), and conjugated ketone (1657 cm^{-1}) functionalities. The NMR spectra were consistent with the presence of a trisubstituted benzene ring; a methyl group attached to an aromatic ring; and an acetyl moiety, a chelated hydroxyl, a conjugated ketone, and a methylene group attached to an oxygenated functionality. Disposition of substituents on the aromatic ring was determined by analysis of the HMBC and NOESY spectra. Thus, the HMBC correlations of the methyl group at δ_C 22 (Ar-CH_3) with H-3″ and H-5″ as well as that of the ketone-carbonyl carbon (C-2′) with H-6″ were consistent with the placement of the methyl and ketone groups at C-4″ and C-1′, respectively. Since the hydroxyl group was chelated, it was readily placed at C-2″, i.e., ortho to the ketone functionality. Furthermore, HMBC correlations of C-1 and C-2′ with H-1′ were in agreement with the acetoxyl and ketone carbonyls being attached to the C-1′ methylene. The NOESY interactions OH/H-3″, H-3″/Ar-CH_3, H-5″/Ar-CH_3, H-1′/H-2, and H-6″/H-1′ provided additional support for these proposals.

To verify the structure of **1** and to obtain additional amounts for biological studies, its synthesis was undertaken. The synthetic path to phytotoxin **1** is outlined in Scheme 1. The general strategy was based on a Fries rearrangement of a suitable acetyl derivative of *m*-cresol (**3**).[10,11] The synthesis started from *m*-cresol, which was treated with Ac_2O to furnish **3**,[12] which was subjected to

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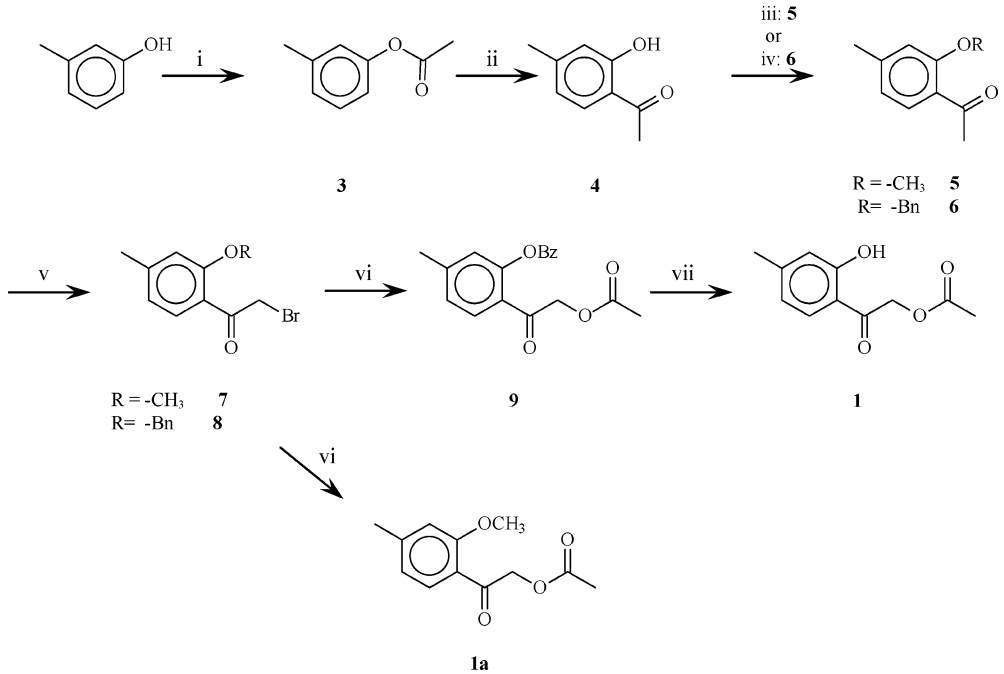
Scheme 1[a]



[a] Conditions: (i) Ac_2O; (ii) $AlCl_3$,CS_2,180 °C; (iii) NaOH, $(CH_3)_2$ SO_4, ice-water bath; (iv) benzyl bromide, K_2CO_3, acetone reflux, 5 days; (v) Br_2, benzene, rt, 4 h; (vi) AcOH, DBU, THF, rt, 2.5 h; (vii) H_2, 10% Pd−C, 60 psi, 60 °C, 5 h.

Fries rearrangement at 180 °C to generate 2-hydroxy-4-methylacetophenone (**4**).[11] The free hydroxyl group of **4** was protected with benzyl bromide to yield 2-benzyloxy-4-methylacetophenone (**6**),[13] which upon α-bromination with Br_2 rendered 2-benzyloxy-ω-bromo-4-methylacetophenone (**8**); the protection of the phenolic group facilitated the α-ketone halogenation reaction. The displacement of bromide from **8** with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU)−CH_3COOH produced ω-acetoxy-2-methoxy-4-methylacetophenone (**9**, 17% of yield), which was catalytically hydrogenated with palladium−charcoal (10% Pd−C) to afford **1** with an overall yield of 11.8%. The methyl derivative **1a** was also synthesized (Scheme 1) using the same approach; in this case the intermediate **4** was subjected to methylation with $(Me)_2SO_4$ to yield 2-methoxy-4-methylacetophenone (**5**),[14] which upon treatment with Br_2 rendered 2-methoxy-ω-bromo-4-methylacetophenone (**7**);[15] finally, treatment of **7** with acetic acid/DBU afforded **1a** (15% of yield). The structures of all the synthetic compounds were elucidated mainly on the basis of their ^1H and ^{13}C NMR spectra as well as MS data.

The phytotoxic effects of **1** and **2** were determined using a Petri dish bioassay.[2,3] Both natural products inhibited radical growth of *Amaranthus hypochondriacus* ($IC_{50} = 3.8 \times 10^{-4}$ and 1.2×10^{-5} M, respectively). The dimer was 10-fold more active than the northymol derivative **1**. *Echinocloa crus-galli* was also sensitive to the treatments with compound **1**. Among the synthetic compounds only the methyl derivative **5** interfered ($IC_{50} = 3.2 \times 10^{-4}$ M) with the growth of seedlings of *Amaranthus*. However, none of the tested compounds affected significantly radical growth of *Medicago sativa*.

The inhibitory effects of **1** and **2** on CaM were also performed to check the relationship between phytotocity and CaM inhibition. This target was selected considering that CaM is a major Ca^{2+}-binding protein that influences a number of important plant growth events through the activation of CaM-dependent enzymes, such as cAMP phosphodiesterase (PDE-1), NAD-kinase, protein phosphatase, and nitric oxide (NO) synthase.[16] Therefore, agents that inhibit the activity of CaM should have an effect on the development of the plant as previously described.[3,4,17] To study if the phytotoxins affected the enzyme regulatory properties of CaM in vitro, their effect on the activity of PDE1 was investigated. PDE1 catalyzes the hydrolysis of cyclic nucleotides to nucleotide monophosphates.[3,4] Since PDE1 is widely used as a tool to discover CaM inhibitors, and in general to demonstrate the activity of CaM in biochemical studies, we assessed the effect of **1** and **2** on this enzyme. The PDE reaction was coupled to the 5′-nucleotidase reaction, and the amount of inorganic phosphate released represented the activity of PDE1. Bovine-brain CaM was used as an activator of the enzyme.[3,4] Natural products **1** and **2** inhibited activation of PDE1 in the presence of CaM with IC_{50} values of 4.4 ± 0.46 and 4.2 ± 0.5 μM, respectively. Their effect was comparable to chlorpromazine ($IC_{50} = 6.8 \pm 1.9$ μM), a well-known CaM inhibitor used as positive control.

Thymol derivatives have been described previously from other Asteraceae such as *Eupatorium fortunei* Turcz.[18] and *Arnica sachalinensis* (Regl.) A. Gray.[9] However, northymol derivatives as **1** are uncommon.

Experimental Section

General Experimental Procedures. Melting point determinations were determined using a Fisher-Johns apparatus and are uncorrected. Optical rotations were recorded on a JASCO DIP 360 digital polarimeter. IR spectra were obtained using KBr disks on a Perkin-Elmer 599B spectrophotometer. UV spectra were recorded on a Shimadzu 160 UV spectrometer in MeOH solution. NMR spectra including COSY, NOESY, HMBC, and HMQC experiments were recorded in $CDCl_3$ on a Varian Unity Plus 500 spectrometer or on a Bruker DMX500 spectrometer at 500 MHz (^1H) or 125 MHz (^{13}C) NMR, using tetramethylsilane (TMS) as an internal standard. MS were obtained on a JEOL JMS-AX505HA mass spectrometer. Column chromatography: silica gel 60 (70−230 mesh, Merck). TLC: Si gel 60 F254 (Merck). Solvents and chemicals were of analytical grade and purchased from Aldrich Company.

Plant Material. The whole plant of *H. schaffneri* was collected from cultivated plants near Ozumba, State of Mexico.

A voucher specimen (Bye and Linares 31018) has been deposited in the National Herbarium (MEXU), Instituto de Biología, UNAM.

Extraction and Isolation. The air-dried plant material (1.6 kg) was ground into a powder and extracted by maceration with CH_2Cl_2−MeOH (1:1) at room temperature. After filtration, the extract was evaporated under reduced pressure to yield 350 g of a green residue, which was subjected to column chromatography over silica gel (818 g) and eluted with a gradient of hexane−CH_2Cl_2 (50:50 → 0:100) and CH_2Cl_2−MeOH (99:1 → 50:50). Fractions of 1 L each were collected and pooled based on TLC profiles to yield 12 major fractions (F1−F12). According, to the Petri dish bioassay fractions F3−F6 concentrated the phytotoxic activity (IC_{50} = 63.09, 52.78, 45.39, and 47.21 μg/mL, respectively). From inactive primary fraction F2 (5 g, eluted with hexane−CH_2Cl_2, 2:3) 49 mg of β-carotene spontaneously crystallized. From primary active fraction F3 (4 g, eluted with hexane−CH_2Cl_2, 3:7) 456 mg of euparine spontaneously crystallized. From active fraction F4 (16.7 g, eluted with hexane−CH_2Cl_2, 1:4) precipitated 147 mg of β-sitosterol. The mother liquors of F4 (16 g) were further chromatographed on a silica gel (269 g) column, eluting with a gradient of hexane−CH_2Cl_2 (10:0 → 1:9) and CH_2Cl_2−MeOH (10:1 → 5:5) to afford eight secondary fractions (F4-1−F4-8). According to the bioautographic assay the strongest activity was in secondary fractions, namely, F4-4 and F4-5. Fraction F4-4 (2.5 g, eluted with hexane−CH_2Cl_2, 7:3) was further resolved on a silica gel (80 g) column, eluting with mixtures of hexane−CH_2Cl_2 (10:0 → 0:10) and CH_2Cl_2−MeOH (10:0 → 0:10) to give 11 tertiary fractions (F4-4-1−F4-4-11). The only active tertiary fraction was F4-4-8 [342 mg, eluted with CH_2Cl_2]. The latter fraction was further purified by column chromatography on silica gel (30 g), eluting with hexane−CH_2Cl_2, 1:1, to afford 8 mg of compound **1**. Column chromatography of the active fraction F4-5 (1.9 g, eluted with hexane−CH_2Cl_2, 6:4) on silica gel (55 g) using CH_2Cl_2 as mobile phase yielded 72 mg of compound **2**.

2′-(2″-Hydroxy-4″-methylphenyl)-2′-oxoethyl acetate (1): colorless crystalline needles, mp 87 °C; UV (CH_2Cl_2) λ_{max} (log ϵ) 328 (3.78), 263 (3.30) nm; IR ν_{max} (KBr) 3500, 2920, 1747, 1657, 1508, 1276, 1228, 1205, 1076, 998 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.24 (3H, s, H-2), 2.36 (3H, s, Ar-CH_3), 5.33 (2H, s, H-1′), 6.73 (1H, ddq, J = 8.1, 1.5, 0.6 Hz, H-5″), 6.83 (1H, dq, J = 1.5, 0.6 Hz, H-3″), 7.49 (1H, d, J = 8.4 Hz, H-6″), 11.6 (1H, s, OH); ^{13}C NMR ($CDCl_3$) δ 20 (C-2), 22 (Ar−CH_3), 64 (C-1′), 119 (C-3″), 115 (C-4″), 121 (C-5″), 128 (C-6″), 149 (C-1″), 162 (C-2″), 170 (C-1), 196 (C-2′); EIMS *m/z* 208 [M]$^+$ (33), 166 (48), 149 (18), 135 (100), 119 (6), 107 (21), 91 (3), 77 (21), 53-(5), 43 (15); HREIMS *m/z* 208.2149 (calcd for $C_{11}H_{12}O_4$, 208.2159).

3-Methylphenyl Acetate (3), 2-Hydroxy-4-methylacetophenone (4), 2-Methoxy-4-methylacetophenone (5), 2-Benzyloxy-4-methylacetophenone (6), and 2-Methoxy-ω-bromo-4-methylacetophenone (7). Compounds **3**−**7** were prepared as previously described[10−15,19,20] (see Supporting Information).

2-Benzyloxy-ω-bromo-4-methylacetophenone (8). Compound **6** (2.0 g, 8.33 mmol) and anhydrous benzene (15 mL) were mixed; then the system was cooled to 0 °C. To this solution was added dropwise, under vigorous stirring, bromine (0.43 mL, 8.33 mmol) dissolved in benzene (10 mL).[20] The reaction mixture was stirred at room temperature for 4 h and then evaporated to dryness to give a solid that was purified by open column chromatography [silica gel, hexane−EtOAc (97:3)]. Compound **8** (0.98 g 40% yield) was obtained as a white solid: mp 84 °C; IR (KBr) ν_{max} 1676, 1601, 1494, 1418, 1267, 1180, 1119, 991 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.40 (3H, s, CH_3), 4.51 (2H, s, −CH_2Br), 5.17 (2H, s, Ar-CH_2O), 6.86 (1H, br d, H-3′), 6.87 (1H, br d, J = 7.2 Hz, H-1′), 7.45−7.39 (5H, m, H-2″−H-6″), 7.76 (1H, d, J = 8.4 Hz, H-6′); ^{13}C NMR ($CDCl_3$) δ 22 (−CH_3), 37.6 (−CH_2Br), 70.9 (ArCH_2O), 113. 3 (C-5′), 122.3 (C-4′), 122.5 (C-3′), 127.8, 128.5, 128.8 (C-2″−C-6″), 131.8 (C-6′), 135.7 (C-1″), 146.0 (C-1′), 158.0 (C-2′), 193 (−CO); FABMS *m/z* 319, 321 [M + H]$^+$ (10, 8), 239 (15), 149 (40), 91 (100), 69 (34), 43 (44).

ω-Acetoxy-2-methoxy-4-methylacetophenone. (1a) In a 50 mL round-bottomed flask was mixed glacial acetic acid (0.20 mL, 4.3 mmol) in dry THF (7 mL) at room temperature. To this solution was added DBU (0.65 mL, 4.3 mmol) under vigorous stirring. After 10 min, ω-bromoketone **7** (0.2 g, 2.88 mmol) dissolved in dry THF (2 mL) was added to the mixture, which was then stirred for 2.5 h.[21] After removal of the solvent in vacuo, the residue was dissolved in CH_2Cl_2 (20 mL) and washed consecutively with saturated Na_2CO_3 solution (2 × 10 mL) and water (2 × 10 mL). The organic fraction was dried over $MgSO_4$, and the solvent was removed in vacuo to give a crude product that was purified by open column chromatography [silica gel, hexane−EtOAc (8:2)]. The desired product **1a** (0.48 g, 76% yield) was obtained as a colorless oil: IR (film) ν_{max} 1735, 1678, 1605, 1404, 1236, 1172, 815 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.21 (3H, s, H-2), 2.38 (3H, s, Ar-CH_3), 3.93 (3H, s, −OCH_3), 5.22 (2H, s, H-1′), 6.78 (1H, br d, J = 8.1 Hz, H-5″), 6.85 (1H, br d, J = 1.2 Hz, H-3″), 7.85 (1H, d, J = 8.4 Hz, H-6″); ^{13}C NMR ($CDCl_3$) δ 20 (C-2), 22 (Ar-CH_3), 55 (−OCH_3), 70 (C-1′), 112 (C-3″), 113 (C-4″), 122 (C-5″), 131 (C-6″), 146 (C-1″), 160 (C-2″), 171 (C-1), 192 (C-2′); EIMS *m/z* 222 [M]$^+$ (15), 207 (19), 197 (2), 195 (1), 161(4), 135 (4), 134 (5), 91 (100).

ω-Acetoxy-2-Benzyloxy-4-methylacetophenone. (9) In a 50 mL round-bottomed flask was mixed glacial acetic acid (0.40 mL, 7.03 mmol) in dry THF (10 mL) at room temperature. To this solution was added DBU (1.05 mL, 7.03 mmol) under vigorous stirring. After 10 min, ω-bromoketone **8** (1.5 g, 4.68 mmol) dissolved in dry THF (2 mL) was added to the mixture, which was then stirred for 2.5 h. After removal of the solvent in vacuo, the residue was dissolved in CH_2Cl_2 (20 mL) and washed consecutively with saturated Na_2CO_3 solution (2 × 10 mL) and water (2 × 10 mL).[21] The organic fraction was dried over $MgSO_4$, and the solvent was removed in vacuo to give a crude product that was purified by open column chromatography [silica gel, hexane−EtOAc (8:2)]. The desired product **1a** (1.15 g, 82.45% yield) was obtained as a white solid: mp 73 °C; λ_{max} (log ϵ) 253 (4.14), 308 (3.7) nm; IR (KBr) ν_{max} 1735, 1678, 1605, 1404, 1236, 1172, 815 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.17 (3H, s, H-2), 2.39 (3H, s, Ar-CH_3), 5.15 (2H, s, CH_2), 5.16 (2H, s, CH_2), 6.86 (1H, br d, J = 1.2 Hz, H-5″), 6.82 (1H, br d, J = 7.8 Hz, H-3″), 7.47−7.26 (5H, m, H-2‴−H-6‴), 7.87 (1H, d, J = 8.1 Hz, H-6″); ^{13}C NMR ($CDCl_3$) δ 20.6 (−CH_3), 21. 9 (−CH_3), 70.1 (CH_2), 70.87 (CH_2), 113.2 (C-5″), 122.2 (C-4″), 122.3 (C-3″), 127.9, 128.5, 128.9 (C-2‴−C-6‴), 131.2 (C-6″), 135.7 (C-1‴), 146.2 (C-1″), 158.8 (C-2″), 170.5 (−CO), 192.2 (−CO); EIMS *m/z* 298 [M]$^+$ (2), 256 (2), 238 (30), 225 (92), 221 (2), 197 (2), 195 (1), 161 (4), 135 (4), 134 (5), 91 (100), 65 (5), 43 (5), 39 (2).

2′-(2″-Hydroxy-4″-methylphenyl)-2′-oxoethyl acetate (1). ω-Acetoxyacetophenone (**9**) (1.0 g, 3.3 mmol) in EtOAc (100 mL) and Pd/C (10%, 0.1 g) were placed in a hydrogenation flask under a stream of anhydrous N_2 gas; the flask was then pressurized with H_2 (60 bar) and shaken at 60 °C for 5 h (TLC control).[15] When the reaction was over, the catalyst was removed by filtration over Celite and the solvent evaporated under reduced pressure to yield **1**, which was purified by open column chromatography [silica gel, hexane−EtOAc (8:2)] to yield 564 mg (57%). The physical and spectroscopy data were identical to those of the natural product.

Phytogrowth-Inhibitory Bioassays. The phytogrowth-inhibitory activity of the crude extract, fractions, and pure compounds was evaluated on seeds of *Amaranthus hypochondriacus, Echinochloa crus-galli,* and *Medicago sativa* using a Petri dish bioassay.[2,3] In addition, a bioautographic phytogrowth-inhibitory bioassay[2,3] was employed to guide secondary fractionation. The seeds of the weeds were purchased from Mercado de Tulyehualco, Mexico City, Mexico. The results were analyzed by ANOVA ($p < 0.05$), and IC_{50} values were calculated by probit analysis based on percent of radicle growth. Samples were evaluated at 10, 100, and 1000 μg mL^{-1}. The bioassays were performed at 28 °C. 2,4-D was used as positive control (IC_{50} values for *A. hypochondriacus, E. crus-galli,* and *M. sativa* were 4 × 10^{-5}, 2 × 10^{-4}, and 0.3 × 10^{-5} M, respectively).

CaM-PDE1 Assay. The CaM-PDE1 assay was performed in a 96-well plate as previously described;[4] the assay is based on the quantification of the amount of inorganic phosphate released from the hydrolysis of AMP by the action of a 5′-nucleotidase from *Crotalus atrox* venom (Sigma). The AMP is generated from cAMP by the action of PDE1. Chlorpromazine was used as a positive control.

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Supporting Information Available: 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 of A. Pérez-Vásquez.
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