CHEMISTRY & BIODIVERSITY

A New Depigmenting-Antifungal Methylated Grindelane from *Grindelia chiloensis*

María de los A. Mesurado,[a] María L. Arias Cassará,[a] Rosana Misico,[b] Alicia Bardón,[a] María I. Ybarra,[a] and Elena Cartagena*[a]

[a]Instituto de Química Orgánica, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Ayacucho, 471, 4000 Tucumán, Argentina, e-mail: ecartagena@fbqf.unt.edu.ar

[b]Departamento de Química Orgánica and UMYMFOR (CONICET-UBA), Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, C1428EGA Ciudad de Buenos Aires, Argentina

The new methylated grindelane diterpenoid, 7β-hydroxy-8(17)-dehydrogrindelic acid (**1b**), together with the known 7α-hydroxy-8(17)-dehydrogrindelic acid (**2a**), 6-oxogrindelic acid (**3a**), 4β-hydroxy-6-oxo-19-norgrindelic (**4a**), 19-hydroxygrindelic acid (**5a**), 18-hydroxygrindelic acid (**6a**), 4α-carboxygrindelic acid (**7a**), 17-hydroxygrindelic acid (**8a**), 6α-hydroxygrindelic acid (**9a**), 8,17-bisnor-8-oxagrindelic acid (**10a**), 7α,8α-epoxygrindelic acid (**11a**), and strictanonic acid (**12a**) as methyl esters were obtained from an Argentine collection of *Grindelia chiloensis* (Cornel.) Cabrera. Their structures and relative configurations were established on the basis of spectroscopic analysis. $CHCl_3$ extract from the aerial parts and their pure compounds were evaluated for their antifungal and depigmenting effects. Methyl ester derivative of **10a** (**10b**) exhibited a remarkable mycelial growth inhibition against *Botritis cinerea* with an IC_{50} of 13.5 μg ml^{-1}. While the new grindelane **1b** exerted a clear color reduction of the yellow-orange pigment developed by *Fusarium oxysporum* against UV-induced damage.

Keywords: *Grindelia chiloensis*, Grindelane diterpenoids and derivatives, Antifungal and depigmenting effects, *Botrytis cinerea*, *Fusarium oxysporum*.

Introduction

Grindelia Wild. genus (Asteraceae: Astereae) comprises approximately 70 species with a highly variable array of ecological forms occurring over a large area of America in diverse neotropical habitats with disjunct distribution in xerophytic or halophytic areas. The genus is characterized by the abundant production of resinous exudates which cover the surface of leaves, stems and involucres of the flower heads.[1] Most of *Grindelia* species are used in folk medicine as antispasmodic and diuretic, among other purposes.[2]

Previous studies found that although all *Grindelia* resins have distinct chemical fingerprint patterns, they are all characterized by the occurrence of labdane-type bicyclic diterpene, called grindelanes.[3] Labdane diterpenoids displayed bioactive properties towards different target organisms like *Tenebrio molitor* larvae species, pathogenic fungi and bacteria.[4] For this reason, these attractive structures are currently objectives for total organic synthesis.[5]

Grindelia chiloensis 'gold button' is a shrub indigenous to Patagonia, Argentina, in the process of domestication as a source of resins to complement the resin production by pines. This woody perennial can accumulate as much as 25% resins (terpenes) in its leaves which increased their thickness and the epicuticular resin production under solar UV-B irradiation. These chemical and structural changes could protect the plant from UV radiation in an environment with maximum daily integrated values of solar UV-B irradiance.[6][7]

Resins are composed of acidic and neutral fractions. Diterpene acids were isolated from the acid fraction; while methyl esters of grindelic acids from the non-acidic fraction; the remainder of neutrals is a mixture of flavonoids and unidentified waxes.[8] Grindelane acids are often isolated as methyl ester derivatives.[9][10] Antifungal activity of *G. chiloensis* compounds has not previously been reported. Thus, the purpose of the present investigation was to inquire into the occurrence of interesting labdane-diterpenoid compounds in an Argentinean collection of this species and determine whether the natural compounds or their methylated derivatives were active against two pathogenic fungi, *Botrytis cinerea*

and *Fusarium oxysporum*, which cause grey-mold and vascular wilt diseases, respectively, and have a negative impact on agriculture, by producing serious economic losses.[11]

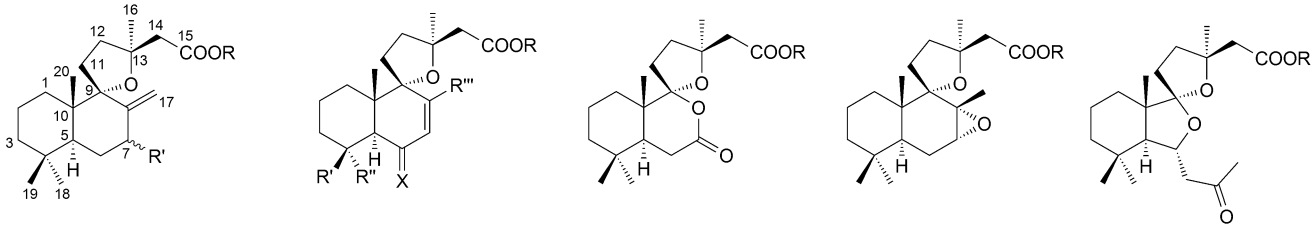
Herein, we quantified *F. oxysporum* pigments by spectroscopy. In addition, the actions of the isolated diterpenoids and their derivatives on the adaptive pigmentation process were also determined.[12][13]

Results and Discussion

The air-dried plant material was ground and then extracted with $CHCl_3$. The extract was subjected to column chromatography (CC) on silica gel. The acid fractions were subsequently methylated and subjected to preparative RP-HPLC to furnish the new grindelane diterpenoid methyl 7β-hydroxy-8(17)-dehydrogrindelate (**1b**) as a methylated derivative of natural product **1a**. The known compounds methyl 7α-hydroxy-8(17)-dehydrogrindelate (**2b**),[9][14][15] 6-oxogrindelic acid (**3a**),[9][15 – 17] methyl 4β-hydroxy-6-oxo-19-norgrindelate (**4b**),[17] methyl 19-hydroxygrindelate (**5b**),[9][14][18] methyl 18-hydroxygrindelate (**6b**),[9][14] methyl 4α-carboxygrindelate (**7b**),[9][17] methyl 17-hydroxygrindelate (**8b**),[9][14 – 16][19][20] methyl 6α-hydroxygrindelate (**9b**),[9][16][21] methyl 8,17-bisnor-8-oxagrindelate (**10b**),[9] 7α,8α-epoxygrindelic acid (**11a**),[14][16][17][22] and methyl strictanoate (**12b**)[9][15][16][23] were also obtained here. Nevertheless, **1b**, **3b** – **5b**, **7b**, **9b** – **12b** are reported for the first time like derivatives of *G. chiloensis* natural products (*Fig. 1*).

The HR-ESI-MS of compound **1b** showed a *quasi*-molecular ion peak at *m/z* 351.25241 ($[M + H]^+$) as well as a $[M + Na]^+$ adduct at *m/z* 373.2349 indicating the molecular formula $C_{21}H_{34}O_4$, which accounted for five degrees of unsaturation. One of them, being an exomethylene group that was inferred from the typical ^{1}H-NMR signals (*Table 1*) at δ(H) 5.18 (*d*, J = 1.5, H_a–C(17)) and δ(H) 4.89 (*d*, J = 1.5, H_b–C(17)). In the ^{13}C-NMR spectrum (*Table 1*), a signal at δ(C) 171.70 was assigned to an ester carbonyl group. Therefore, the molecule possesses three rings. The ^{13}C-NMR and HSQC-DEPT spectra showed the presence of five methyl, eight methylene, two methine groups, and six quaternary C-atoms. The ^{13}C-NMR shifts of three of them at δ(C) 70.56, 91.92 and 81.74 indicated that they are linked to O-atoms. One of two must be part of an ether bridge connecting two fully substituted C-atoms, and the remaining O-atom must be a hydroxy according to the FT-IR absorption at 3480 cm^{-1}.

The presence of a secondary alcohol was evident by ^{1}H-NMR resonance signals at δ(H) 4.52 (*dd*, J = 11.5, 5.5, H_α–C(7)) assignable to a geminal H-atom bonded to C–OH. A signal at δ(C) 70.56 in ^{13}C-NMR, and the fragment ion at *m/z* 333.2428 ($[M + H–H_2O)$ in the HR-ESI-MS spectrum, unambiguously confirm the presence of a OH group. The location of the OH group at C(7) was established by the HMBC cross-peaks observed between H–C(7) (δ(H) 4.52) with C(5), C(6), and C(17). The β-orientation of the OH group was established from the ROESY spectrum, which showed strong correlations between H_α–C(7) and H_α–C(6). In addition, the large coupling constant between the H_α–C(7) and H_β–C(6) (J = 11.5 Hz) confirmed an anti-periplanar position of H-atoms and the configuration proposed (*Fig. 2*). A pair of doublets appeared at δ(H) 2.56 and 2.50 (J = 14.0 Hz), indicating the presence of a methylene group (H_a–C(14) and H_b–C(14)).



1a R = H, R' = β-OH
1b R = Me, R' = β-OH
2a R = H, R' = α-OH
2b R = Me, R' = α-OH

3a R = H, R',R'',R''' = Me, X = O
3b R,R',R'',R''' = Me, X = O
4a R = H, R' = OH, R'',R''' = Me, X = O
4b R,R'',R''' = Me, R' = OH, X = O
5a R = H, R' = CH_2OH, R'',R''' = Me, X = H_2
5b R,R'',R''' = Me, R' = CH_2OH, X = H_2
6a R = H, R'R''' = Me, R'' = CH_2OH, X = H_2
6b R,R',R''' = Me, R'' = CH_2OH, X = H_2
7a R = H, R',R''' = Me, R'' = COOH, X = H_2
7b R,R',R''' = Me, R'' = COOH, X = H_2
8a R = H, R',R'' = Me, R''' = CH_2OH, X = H_2
8b R,R',R'' = Me, R''' = CH_2OH, X = H_2
9a R = H, R',R'',R''' = Me, X = α-OH
9b R,R',R'',R''' = Me, X = α-OH

10a R = H
10b R = Me

11a R = H
11b R = Me

12a R = H
12b R = Me

Figure 1. Chemical structures of compounds **1** – **12**.

Table 1. ^{1}H-, ^{13}C- and HMBC NMR data for compound **1b** ($CDCl_3$)

Position	δ(H)	δ(C)	HMBC (H → C)
1	1.62 – 1.52 (*m*, H_β), 1.40 – 1.30 (*m*, H_α),	32.04 (*t*)	
2	1.58 – 1.54 (*m*, H_α), 1.53 – 1.48 (*m*, H_β)	19.13 (*t*)	
3	1.43 – 1.32 (*m*, H_β), 1.25 – 1.15 (*m*, H_α)	41.71 (*t*)	
4		33.52 (*s*)	
5	1.72 (*dd*, *J* = 13.0, 3.0, H_α)	44.62 (*d*)	3, 4, 7, 9, 20
6	2.02 (*ddd*, *J* = 12.0, 5.5, 3.0, H_α), 1.20 (*dd*, *J* = 13.0, 12.0, 11.5, H_β)	33.56 (*t*)	5, 7 7
7	4.52 (*dd*, *J* = 11.5, 5.5, H_α)	70.56 (*d*)	5, 6α, 6β, 17
8		153.09 (*s*)	
9		91.92 (*s*)	
10		41.68 (*s*)	
11	2.13 (*ddd*, *J* = 13.0, 8.0, 7.5, H_α), 1.96 (*ddd*, *J* = 13.0, 11.0, 3.5, H_β)	26.09 (*t*)	9, 12 11, 13, 14
12	2.19 – 2.09 (*m*, H_β), 1.73 (*ddd*, *J* = 13.0, 11.0, 8.0, H_α)	36.73 (*t*)	 9, 11
13		81.74 (*s*)	
14	2.56 (*d*, *J* = 14.0, H_a), 2.50 (*d*, *J* = 14.0, H_b)	46.84 (*t*)	12, 13, 15, 16
15		171.70 (*s*)	
16	1.30 (*s*)	27.21 (*q*)	
17	5.18 (*d*, *J* = 1.5, H_a), 4.89 (*d*, *J* = 1.5, H_b)	103.52 (*t*)	7, 8, 9 7, 9
18	0.93 (*s*)	33.42 (*q*)	3, 4, 5, 19
19	0.82 (*s*)	21.94 (*q*)	3, 4, 5
20	0.76 (*s*)	17.10 (*q*)	1, 5, 9, 10
MeO	3.66 (*s*)	51.42 (*q*)	

^{1}H- and ^{13}C-NMR spectra were recorded at 500 and 125 MHz, respectively.

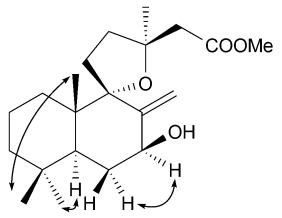


Figure 2. Key ROESY observed for compound **1b**.

The HMBCs between H–C(14) and C(12), C(13), C(15), and C(16) supported this assignment. The position of the exomethylene group (C(8), C(17)) was suggested in the HMBC spectrum (*Table 1*) from the following correlations: $=CH_2$(17) (δ(H)5.18) with C(7), C(8), and C (9), and the resonance signal at H–C(7) (δ(H) 4.52) with C(5), C(6), and C(17). The β-orientation of Me(20) was determined by the ROESY experiment, which showed a strong correlation between Me(20) and Me(19). The α-orientation of Me(16) was deduced from the same spectrum, which showed strong correlations between Me(16) and both H–C(5) and H–C(12).

Total assignments of **1b** were achieved by data from ^{1}H-NMR, ^{1}H,^{1}H-COSY, HMQC, HMBC, ROESY and ^{13}C-NMR experiments. Therefore compound **1b** was identified as methyl 7β-hydroxy-8(17)-dehydrogrindelate.

Compound **2b** is the C(7)-epimer of **1b** as was deduced from the corresponding ^{1}H-NMR data. NMR spectroscopic profiles were quite similar to those of **1b** with the only difference that the signal at δ(H) 4.52 was a *doublet* (*J* = 10.0, C(7)), which is consistent with the β-orientation of H-atom at C(7).

Antifungal Activity on B. cinerea

The $CHCl_3$ sub-extract (SE) displayed a significant growth-inhibition for *B. cinerea*. The antifungal assay on solid culture media showed that SE inhibited 69% mycelial growth at 1000 μg ml^{-1}, while the inhibitory effects at 500 and 250 μg ml^{-1} were 41% and 27%, respectively (*Table 2*).

Antifungal effects of pure grindelanes (**3a**, **11a**, **1b** – **7b**, **10b** – **12b**) in a microtiter assay indicated

that all compounds had differential activity. Compounds **1b** – **3b**, **5b** – **6b**, **10b** and **12b** were active at 100 μg ml^{-1} (42 – 89% growth inhibition). The most active compound was **10b** even at 50 μg ml^{-1} with 84% growth inhibition (*Fig. 3*) and IC_{50} value of 13.5 ± 1.9 μg ml^{-1}.

It is important to note that the grindelane acids **3a** and **11a** exerted strong stimulations on *B. cinerea* growth (66% and 74%, respectively) at 50 μg ml^{-1}, which would be promissory to improve some effluent treatments or for the removal of environmental and industrial pollutants, such as lignins, and phenolic

Table 2. Mycelial growth inhibition of *Botritis cinerea* and *Fusarium oxysporum* for the $CHCl_3$ extract of *Grindelia chiloensis*

Concentration [μg ml^{-1}]	*B. cinerea*			*F. oxysporum*		
	Control radial growth [cm]	Radial growth[a] [cm]	% Growth Inhibition	Control radial growth [cm]	Radial growth [cm]	% Growth inhibition
1000	9.0 ± 0.1	2.8 ± 0.1 *a*	69.2 ± 1.3 *a*	9.0 ± 0.1	5.4 ± 0.1 *a*	40.2 ± 0.8 *a*
500	9.0 ± 0.1	5.3 ± 0.1 *b*	40.8 ± 1.1 *b*	9.0 ± 0.1	7.4 ± 0.1 *b*	17.2 ± 0.9 *b*
250	9.0 ± 0.1	6.5 ± 0.1 *c*	27.3 ± 1.2 *c*	9.0 ± 0.1	8.1 ± 0.1 *c*	10.3 ± 1.3 *c*

[a] Mean ± SD. Means followed by the different letter are significantly different ($P < 0.05$, LSD *Fisher* test).

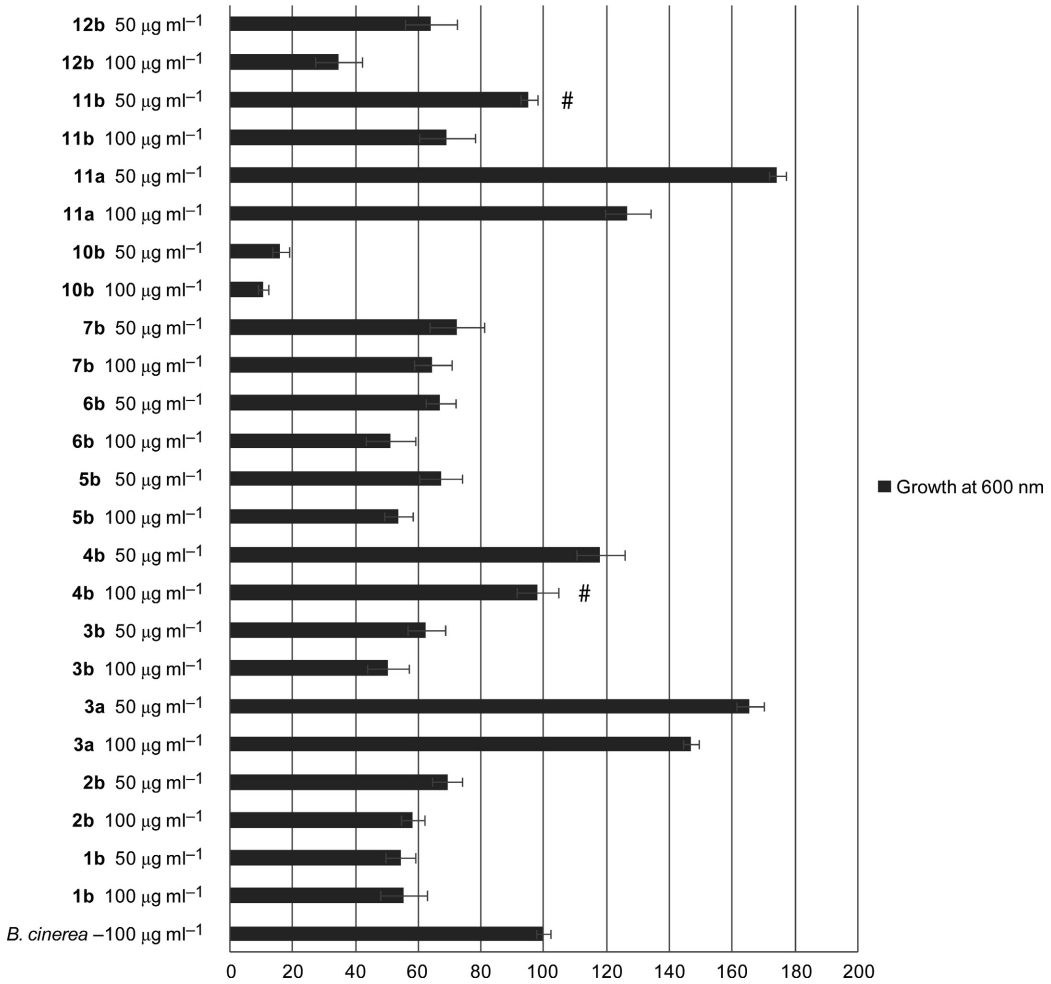


Figure 3. Percentage of *Botrytis cinerea* growth after 48 h of treatment with 100 and 50 μg ml^{-1} of compounds **1b** – **7b**, **10b** – **12b**, **3a** and **11a**. Means for percent are listed for each concentration ($n = 4$) ± standard deviation. The antifungal activity of compounds was compared with the same concentrations of the commercial fungicide, azoxystrobin (100% inhibition at 100 μg ml^{-1}. All samples are significantly different to the control ($P < 0.05$, LSD *Fisher* test) except for bars with #.

compounds mediated by this fungus. Most of the enzyme laccases characterized so far have been derived from efficient lignin degraders like white-rot fungi *B. cinerea*.[24] The lipophilic properties of these compounds were lower than their methylated derivates with theoretical partition coefficient o/w values of 3.8156 for **3a**, and 4.6256 for **11a**, while **3b** was 4.279 and 5.089 for **11b** (this descriptor was calculated using Chem 3D Ultra after minimizing energy of both compounds by the semi-empirical MM2 Program). These results suggested that slight changes in hydrophilic-lipophilic balance exerted opposite activity. Indeed, molecular dynamics studies determined that plant molecules with a hydrophilic head group and a hydrocarbon tail or skeleton (mimicking phospholipids) have a wide variety of effects on the membrane, ranging from stabilization to violent phase transition and disruption, depending on hydrocarbon chain and concentration.[25] This behaviour is consistent with previous publications that demonstrated that small chemical changes on the terpenoid skeleton exert microbial inhibition or stimulation.[26][27] Grindelane acid methyl esters, more lipophilic than acids, could be cell membrane disruptors as are other lipophilic compounds.[28]

Antifungal and Antipathogenic Effects against F. oxysporum

$CHCl_3$ Sub-extract that inhibits 40.0% *F. oxysporum* growth at 1000 μg ml^{-1}, displayed lower inhibitions at 500 and 250 μg ml^{-1} (17.2% and 10.3%, respectively). The inhibitory effects were also dose-depending, coherently with its effect against *B. cinerea* (*Table 2*).

Compounds **2b**, **3b**, **6b**, **7b**, **11b** and **12b** exerted moderate activity on the mycelial development reduction (34 – 44%) at 100 μg ml^{-1} being **7b** and **2b** the most active (44% and 42%, respectively). It is important to point out that stimulant effects were not observed in any experiments (*Fig. 4*).

A differential depigmenting activity was observed in all cases; except for compound **10b** at 50 μg ml^{-1}. Particularly, methylated grindelane **1b** exerted a clear reduction of VIS absorption of the yellow-orange pigment developed by *F. oxysporum* (48%, see the *Supplementary Material*), in the same way as compounds **2b**, **3b, 6b, 7b**, **11b**, and **12b** do (28 – 48%). Their effects would obey to a growth inhibition mode of action, except for **1b** (C(7)-epimer of **2b**), which was exclusively an antipathogenic agent. This lack of correspondence could be due to another mechanism involved in the depigmenting process. Indeed, phytotoxic naphthoquinone pigments from *F. oxysporum* were previously isolated from citrus fibrous roots[29] and these privileged structures for *Fusarium* species can attack plants, fungi and bacteria.[30 – 32] This virulence factor might help the fungus to counteract competitor microorganisms or might play a role in the natural infection process.

Conclusions

Due to the depigmentation process mediated by grindelane compounds, the assayed phytopathogenic fungi become more sensitive to predators and oxidative stress by UV light. An interesting correlation between pigment decrease mediated by plant grindelabdanes or their methylated derivatives, and *F. oxysporum* pathogenicity could be proved *in situ* in future investigations.

Experimental Section

General

For thin layer chromatography (TLC), pre-coated SiO_2 plates (*Merck, Kieselgel 60* F_{254}) were employed. Spots on the plates were detected using *Godin*'s reagent followed by heating at 120 °C.[33] SiO_2 *60* (*Merck*, 70 – 230 mesh) was used for CC. For HPLC separations of mixtures, Gilson apparatus equipped with *Gilson* pump model 305 and Gilson refractive index detector model *133* was used. Columns: (*A) Phenomenex Luna RP-18* (5 μm, 10 mm i.d. × 250 mm) and (*B*) *Phenomenex Luna RP-8* (5 μm, 10 mm i.d. × 250 mm). Retention time was measured from the injection time. Optical rotations were measured on a *HORIBA SEPA-300* high-sensitive polarimeter with $CHCl_3$ as a solvent. UV spectra were recorded on a *Shimadzu UV/VIS 160 A* spectrophotometer. FT-IR spectra were measured on a *FT-PerkinElmer-1600* spectrophotometer; $\tilde{\nu}$ in cm^{-1}. ^{1}H- and ^{13}C-NMR spectra were recorded on a *Bruker Avance II 500* NMR spectrometer (^{1}H at 500.13 MHz, ^{13}C at 125.77 MHz); δ in ppm relative to Me_4Si as internal standard, *J* in Hz. EI-MS and CI-MS were obtained on a *Finnigan Polaris Q* spectrometer; in *m/z* (rel. %).

Plant Material

Grindelia chiloensis was collected in January 2004 at Chubut Province, Argentina. A voucher specimen (LIL No. 607200) was deposited with the Herbarium of the Fundation Miguel Lillo, Tucumán, Argentina.

Extraction and Isolation

The air-dried plant material (1 kg) was ground and then extracted (3 × 1300 ml) with $CHCl_3$ at r.t. for

6 days. The extract was concentrated *in vacuo* to give 239 g (yield 23.9%) of crude extract which was suspended in EtOH (2000 ml) at 55 °C, diluted with H_2O (1500 ml), and extracted successively with hexane (3 × 1200 ml) and $CHCl_3$ (3 × 1200 ml). The $CHCl_3$ extract on evaporating at reduced pressure furnished a residue (104 g, yield 10.4%) which was suspended in AcOEt and separated into neutral and acidic fractions using 5% aq. $NaHCO_3$ followed by neutralization of the alkaline solution with 10% aq. HCl. The acidic fraction was extracted with $CHCl_3$ (3 × 110 ml), washed with H_2O, dried (Na_2SO_4), filtered and concentrated *in vacuo* to furnished 23 g of $CHCl_3$ extract. This extract was subjected to CC on silica gel (630 g) with $CHCl_3$ containing increasing amounts of AcOEt (0 – 100%) and further, AcOEt containing increasing amounts of MeOH (0 – 4%). Fractions of similar composition were pooled on the basis of TLC analysis. Fractions eluted with a mixture of $CHCl_3$/AcOEt 75:25 were combined (*Fr. 1*, 395 mg) and a portion (130 mg) was submitted to a RP-HPLC process (Column *A*, MeOH/H_2O 72:28, 2.2 ml min^{-1}) to yield compounds **3a** (6.9 mg; t_R 43 min) and **11a** (13 mg; t_R 53 min). Fractions eluted with a mixture of $CHCl_3$/AcOEt 65:35 were combined (*Fr. 2*, 1.3 g). Fractions eluted with a mixture of $CHCl_3$/AcOEt/MeOH 1:1:0 → 0:0.95:0.5 were combined (*Fr. 3*, 3.8 g). These two later fractions showed a complex mixture of acid by infrared spectroscopy which was transformed to the methyl esters by treatment with ethereal

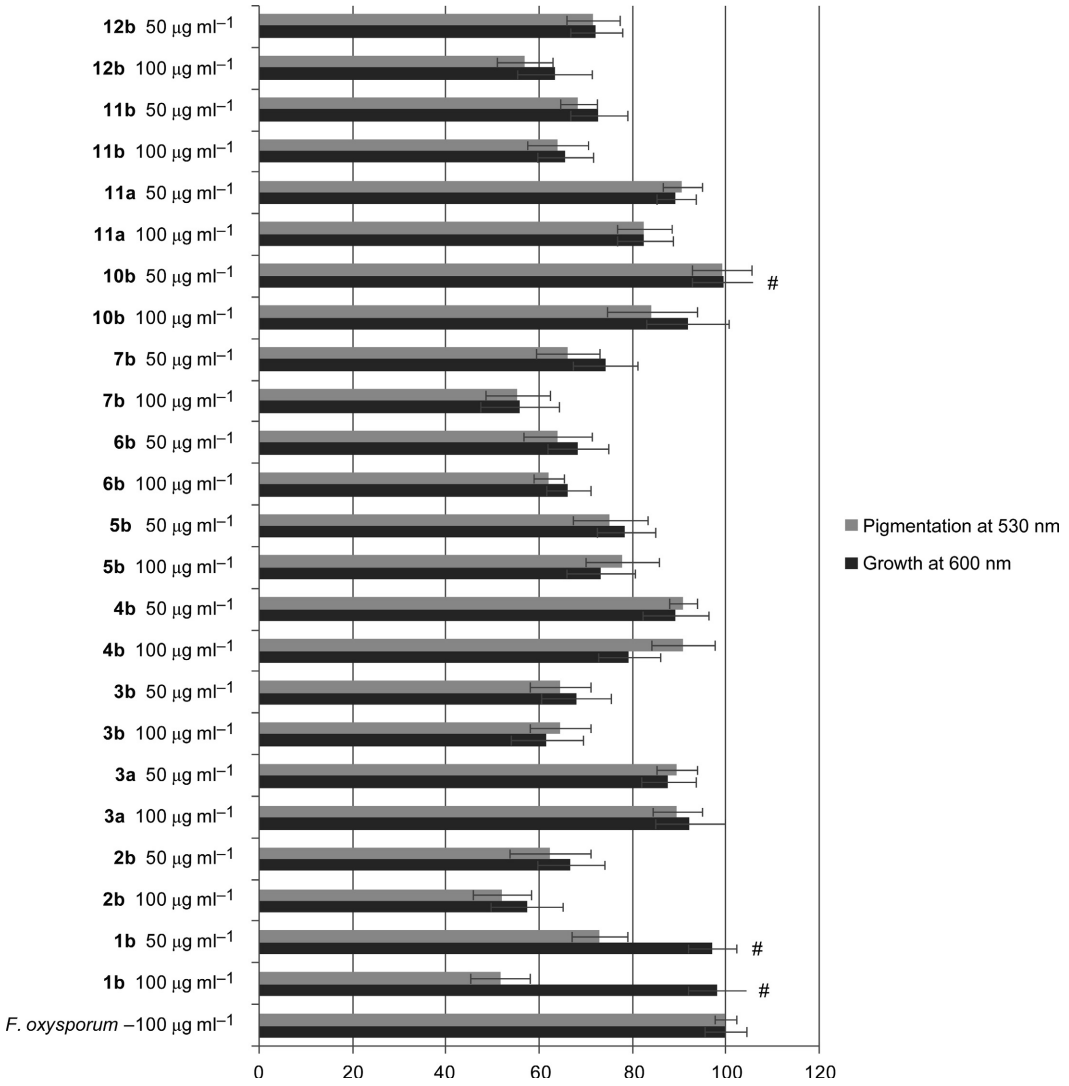


Figure 4. Percentage of growth and pigmentation of *Fusarium oxysporum* after 48 h of treatment with 100 and 50 μg ml^{-1} of compounds **1b** – **7b**, **10b** – **12b**, **3a** and **11a**. Means for percent are listed for each concentration ($n = 4$) ± standard deviation. The antifungal activity of compounds was compared with the same concentrations of the commercial fungicide, imazalil (99% inhibition at 100 μg ml^{-1}). All experiments are significantly different to the corresponding control ($P < 0.05$, LSD *Fisher* test) except for bars with #.

diazomethane. A portion (244 mg) of methyl ester obtained from *Fr. 2* (1.3 g) was chromatographed on RP-HPLC (Column *A*, MeOH/H_2O 85:15, 2.2 ml min^{-1}) to yield **4b** (26.0 mg; t_R 11 min), **5b** (4.7 mg; t_R 15 min), **10b** (13.7 mg; t_R 18 min), a mixture of two compounds (52 mg; t_R 21 min), **1b** (6.1 mg; t_R 25 min), and **2b** (9.3 mg; t_R 30 min). The mixture was purified by RP-HPLC (Column *A*, MeOH/H_2O 75:25, 2.0 ml min^{-1}) to yield **4b** (4.7 mg; t_R 33 min) and **12b** (13.7 mg; t_R 35 min). A portion (374 mg) of methyl ester obtained from *Fr. 4* (8 g) was chromatographed on RP-HPLC (Column *A*, MeOH/H_2O 82:18, 2.0 ml min^{-1}) to yield **6b** (19.4 mg; t_R 37 min), a mixture of two compounds (t_R 39 min), **1b** (1.2 mg; t_R 42 min), **7b** (3.6 mg; t_R 45 min), and **2b** (11.7 mg; t_R 52 min). The mixture was purified by RP-HPLC (Column *B*, MeOH/H_2O 78:22, 2.0 ml min^{-1}) to yield **8b** (3.6 mg; t_R 42 min) and **9b** (1.2 mg; t_R 47 min).

Methyl 7β-Hydroxy-8(17)-dehydrogrindelate (**1b**). Pale yellow oil. $[\alpha]_D^{20}$ = +9.9 (*c* = 0.015, $CHCl_3$). FT-IR ($CHCl_3$): 3480 (OH), 1738 (COOMe), 1092 (COC). ^{1}H- and ^{13}C-NMR: see *Table 1*. EI-MS: 334 (68, M^+), 319 (1, $[M - Me]^+$), 210 (100, $[M - RDA]^+$. HR-ESI-MS: 351.2524 (32, $[M + H]^+$, $C_{21}H_{35}O_4^+$; calc. 351.2526).

Methyl 7α-Hydroxy-8(17)-dehydrogrindelate (**2b**). Pale yellow oil. $[\alpha]_D^{20}$ = −5.1 (*c* = 0.005, $CHCl_3$). ^{1}H-NMR data correlated with literature data.[9][14][15] ^{13}C-NMR data correlated with literature data.[34]

6-Oxogrindelic Acid (**3a**). Pale yellow oil. $[\alpha]_D^{20}$ = −45.3 (*c* = 0.02, $CHCl_3$). ^{1}H-NMR (300 MHz, $CDCl_3$): 5.73 (*s*, H–C(7)); 2.73 (*d*, *J* = 15.0, H_a–C(14)); 2.70 (*s*, H–C(5)); 2.61 (*d*, *J* = 15.0, H_b–C(14)); 2.24 (*ddd*, *J* = 12.0, 11.0, 8.0, H_a–C(11)); 2.20 (*ddd*, *J* = 12.0, 11.0, 8.0, H_a–C(12)); 2.07 (*dd*, *J* = 12.0, 11.4, H_b–C(11)); 2.03 (*dd*, *J* = 12.0, 3.5, H_b–C(12)); 1.98 (*d*, *J* = 1.0, H–C(17)); 1.75 (*dd*, *J* = 12.5, 3.5, H_β–C(1)); 1.62 (*dt*, *J* = 13.5, 3.5, H_α–C(2)); 1.55 (*m*, H_β–C(2)); 1.51 (*dd*, *J* = 12.5, 1.5, H_α–C(1)); 1.47 (*s*, Me(16)); 1.37 (*dd*, *J* = 13.0, 1.5, H_β–C(3)); 1.17 (*dd*, *J* = 13.0, 3.5, H_α–C(3)); 1.19 (*s*, Me(18)); 1.12 (*s*, Me(19)); 0.97 (*s*, Me(20)). ^{13}C-NMR data correlated with literature data.[34][35] CI-MS: 335 (68, $[M + H]^+$), 320 (1, $[M + H - Me]^+$), 317 (12, $[M + H - H_2O]^+$), 292 (1, $[M + H - CO]^+$), 210 (100, $[M + H - C_9H_{16}]^+$).

Methyl 4β-Hydroxy-6-oxo-19-norgrindelate (4b). Pale yellow oil. $[\alpha]_D^{20}$ = −46.8 (*c* = 0.006, $CHCl_3$). ^{1}H- and ^{13}C-NMR data correlated with literature data.[17] EI-MS: 350 (31, M^+), 332 (100, $[M - H_2O]^+$), 277 (4, $[M - CH_2COOMe]^+$), 259 (42, $[M - H_2O - CH_2COOMe]^+$), 224 (21, $[M - C_8H_{14}O]^+$).

Methyl 19-Hydroxygrindelate (5b). Pale yellow oil. $[\alpha]_D^{20}$ = −11.2 (*c* = 0.004, $CHCl_3$). ^{1}H- and ^{13}C-NMR correlated with literature data.[9][18]

Methyl 18-Hydroxygrindelate (6b). Pale yellow oil. $[\alpha]_D^{20}$ = −89.3 (*c* = 0.005, $CHCl_3$). FT-IR ($CHCl_3$): 3430 (OH), 1737 (COOMe), 1092 (COC). ^{1}H-NMR (500 MHz, $CDCl_3$): 5.50 (*dd*, *J* = 3.0, 1.0, H–C(7)); 3.68 (*s*, MeO); 3.38 (*d*, *J* = 10.5, H_a–C(18)); 3.20 (*d*, *J* = 10.5, H_b–C(18)); 2.76 (*d*, *J* = 14.0, H_a–C(14)); 2.62 (*d*, *J* = 14.0, H_b–C(14)); 2.24 (*ddd*, *J* = 11.0, 11.0, 8.0, H_a–C(12)); 2.09 – 2.05 (*m*, H_a–C(11)); 2.04 (*dt*, *J* = 11.0, 11.0, 1.0, H_β–C(3)); 2.01 – 1.98 (*m*, H_α–C(6)); 1.92 – 1.83 (*m*, H–C(5)); 1.89 – 1.82 (*m*, H_β–C(6), H_b–C(12); 1.83 – 1.79 (*m*, H_b–C(11)); 1.78 (*d*, *J* = 1.0, H–C(17)); 1.72 – 1.66 (*m*, H_α–C(2)); 1.58 – 1.53 (*m*, H_β–C(2)); 1.51 – 1.43 (*m*, H_α–C(1); H_β–C(1)); 1.46 – 1.40 (*m*, H_α–C(3)); 1.35 (*s*, Me(16)); 0.91 (*s*, Me(19)); 0.88 (*s*, Me(20)). ^{13}C-NMR (125 MHz, $CDCl_3$): 171.91 (C(15)); 135.12 (C(8)); 126.02 (C(7)); 90.46 (C(9)); 81.74 (C(13)); 72.55 (C(18)); 51.45 (C(OMe)); 47.97 (C(14)); 40.51 (C(10)); 38.30 (C(12)); 37.86 (C(4)); 37.15 (C(5)); 35.50 (C(3)); 32.54 (C(1)); 28.49 (C(11)); 27.40 (C(16)); 24.21 (C(6)); 21.30 (C(17)); 18.09 (C(2)); 17.97 (C(19)); 17.27 (C(20)). CI-MS: 351 (56, $[M + H]^+$), 333 (10, $[M + H - H_2O]^+$), 321 (15, $[M + H - H_2O - CO]^+$), 210 (100, $[M + H - RDA]^+$).

Methyl 4α-Carbomethoxigrindelate (7b). Pale yellow oil. $[\alpha]_D^{20}$ = −69.3 (*c* = 0.02, $CHCl_3$). FT-IR ($CHCl_3$): 1728 (COOMe), 1245 (C–O), 1093 (COC), 992 (C=C). ^{1}H- and ^{13}C-NMR correlated with literature data.[9][36] CI-MS: 379 (45, $[M + H]^+$), 361 (20, $[M + H - H_2O]^+$), 318 (6, $[M + H - CO_2Me]^+$), 321 (15, $[M + H - H_2O - CO]^+$), 210 (100, $[M + H - RDA]^+$).

Methyl 17-Hydroxygrindelate (8b). Pale yellow oil. ^{1}H-NMR (500 MHz, $CDCl_3$): 5.90 (*dd*, *J* = 4.0, 3.0, H–C(7)); 4.16 (*dq*, *J* = 12.5, 3.5, H–C(17)); 2.76 (*d*, *J* = 14.0, H_a–C(14)); 2.58 (*d*, *J* = 14.0, H_b–C(14)); 2.23 – 2.20 (*m*, H_a–C(11)); 2.20 – 2.16 (*m*, H_α–C(6)); 2.12 – 2.08 (*m*, H_a–C(12)); 1.94 – 1.87 (*m*, H_β–C(6)); 1.92 – 1.88 (*m*, H_b–C(12)); 1.78 – 1.72 (*m*, H_β–C(1)); 1.75 (*dd*, *J* = 12.0, 5.5, H–C(5)); 1.59 – 1.55 (*m*, H_α–C(1)); 1.35 (*s*, Me(16)); 0.92 (*s*, Me(19)); 0.90 (*s*, Me(18)); 0.82 (*s*, Me(20)); 3.68 (*s*, MeO). ^{13}C-NMR (125 MHz, $CDCl_3$): 171.81 (C(15)); 139.33 (C(8)); 130.29 (C(7)); 90.07 (C(9)); 81.73 (C(13)); 65.34 (C(17)); 51.51 (MeO); 47.60 (C(14)); 43.00 (C(5)); 41.89 (C(3)); 40.72 (C(10)); 38.20 (C(11)); 35.35 (C(18)); 33.54 (C(4)); 32.50 (C(1)); 27.61 (C(16)); 27.42 (C(12)); 24.14 (C(6)); 22.04 (C(19)); 18.62 (C(2)); 16.78 (C(20)).

Methyl 6α-Hydroxygrindelate (9b). Pale yellow oil. ^{1}H- and ^{13}C-NMR correlated with literature data.[9][21][34]

Methyl 8,17-Bisnor-8-oxagrindelate (10b). Pale yellow oil. $[\alpha]_D^{20}$ = −47.1 (*c* = 0.04, $CHCl_3$). ^{1}H- and ^{13}C-NMR correlated with literature data.[33][37] EI-MS: 338 (100, M^+), 294 (8, $[M - CO_2]^+$), 265 (8, $[M - CH_2CO_2Me]^+$), 173 (62, $C_{10}H_{17}O_3^+$), 109 (82, $C_8H_{13}^+$).

7α,8α-Epoxygrindelic Acid (11a). Pale yellow oil. $[\alpha]_D^{20}$ = +35.6 (*c* = 0.006, $CHCl_3$). ^{1}H-NMR: ($CDCl_3$, 300 MHz): 10.63 (*s*, H–C(15)); 3.21 (*d*, *J* = 2.5, H–C(7)); 2.73 (*d*, *J* = 12.5, H_b–C(14)); 2.57 (*d*, *J* = 12.5, H_a–C(14)); 2.19 (*ddd*, *J* = 6.6, 6.5, 6.7, H_α–C(6)); 2.15 (*dd*, *J* = 10.0, 2.5, H_a–C(11)); 2.08 (*dd*, *J* = 13.0, 2.5, H_b–C(12)); 2.00 (*dd*, *J* = 13.0, 10.0, 2.5, H_a–C(12)); 1.95 (*dd*, *J* = 10, 2, H_b–C(11)); 1.75 (*ddd*, *J* = 6.6, 6.5, 6.7, H_β–C(6)); 1.52 – 1.49 (*m*, H–C(2)); 1.51 – 1.40 (*m*, H_α–C(1)); 1.50 (*dd*, *J* = 13.0, 6.0, H–C(5)); 1.47 (*s*, Me(17)); 1.45 – 1.43 (*m*, H_β–C(1)); 1.40 – 1.37 (*m*, H_β–C(3)); 1.37 (*s*, Me(16)); 1.15 – 1.10 (*m*, H_α–C(3)); 0.90 (*s*, Me(19)); 0.88 (*s*, Me(18)); 0.87 (*s*, Me(20)). ^{13}C-NMR correlated with literature data.[38] CI-MS: 337 (100, $[M + H]^+$), 319 (50, $[M + H - H_2O]^+$), 301 (10, $[M + H - 2H_2O]^+$), 212 (20, $[M + H - RDA]^+$).

Methyl Strictanoate (12b). Pale yellow oil. ^{1}H- and ^{13}C-NMR correlated with literature data.[9][33][35]

Fungi and Media

Fusarium oxysporum isolated from tobacco, and *B. cinerea* from strawberry which were indigenous strains isolated by Cátedra of Fitopatologia, Facultad de Agronomía y Zootecnia, Universidad Nacional de Tucumán, Tucumán, Argentina. Strains were maintained on potato glucose agar 2% as culture media (PGA, *Britania*®). Fungi were grown in 9-cm *Petri* dishes and incubated in a growth chamber at 21 – 23 °C, under cool-white fluorescent lights with a 12-h photoperiod.

Agar Dilution Assay

Growth inhibition of the $CHCl_3$ sub-extract was evaluated in an agar dilution assay. The $CHCl_3$ sub-extract of *G. chiloensis* was dissolved in AcOEt, and a proper dilution was aseptically added to the PGA medium at 45 °C to obtain a final concentration of 1000, 500, and 250 μg ml^{-1}. The AcOEt concentration in the final soln. was adjusted to 2%. The medium with and without test substance was poured into 6-cm diameter *Petri* dishes. Dishes were left open in a laminar-flow hood for 45 min to remove AcOEt. Fungal inoculum discs of 3-mm diameter cut from the periphery of growing mycelium of filamentous fungi were placed aseptically on the center of each *Petri* dish, sealed with *Parafilm*® and incubated in a growth chamber as described previously for fungal growth. The growth rate was determined by measuring daily colony diameter (4 days for *B. cinerea* and 6 days for *F. oxysporum*). At this time, the percentage growth inhibition in comparison with the negative control was calculated for each fungus. Three replicates were used for each concentration. The data were submitted to ANOVA, and the mean values were compared by using the *Fisher* test ($P < 0.05$). Conidia were harvested from 7 – 10-day-old culture by flooding tubes with sterile liquid media and dislodging conidia by softly brushing the colonies with an l-shaped glass rod. Conidial suspensions were filtered through sterile gauze to remove mycelia. Conidia concentrations were determined spectrophotometrically at 600 nm (70% transmittance; conidia concentration of 10^6 conidia ml^{-1}).

Microtiter Assays

The antifungal activity of pure compounds was evaluated with the broth microdilution method using malt-peptone-glucose liquid medium (*Britania*®) for both fungi. The assay was carried out in 96-well flat-bottomed microtiter plates. Stock solns. of **1b** – **7b** and **10b** – **12b** in AcOEt were diluted with liquid medium to give serial twofold dilutions resulting in concentrations from 1.56 to 100.00 μg ml^{-1}. The final concentration of AcOEt in the assay did not exceed 2%. Using a micropipet, an inoculum of 10 ml of the spore suspension was added to each liquid medium well. Negative control wells received medium and inoculum. The liquid medium without test compounds was used as a blank control. The plates were covered with a plastic lid and incubated in a growth chamber as described previously for fungal growth. The growth was evaluated by measuring the absorbance of each well at 600 nm, using a microtiter plate reader (*Power Wave XS2, Biotek*, VT, USA). The plates were agitated and read immediately thereafter. Mean absorbance values were used to evaluate fungal growth at 24, 48, and 72 h; 48 h was selected as the most representative time point.

The percentage of growth inhibition and pigmentation was expressed as the mean of values obtained in eight independent experiments. For comparison, the same concentrations of the commercial fungicides, imazalil (*Fungaflor, BASF*, Buenos Aires, Argentina) and azoxystrobin (*Amistar, Syngenta*, Buenos Aires, Argentina) were included in the assay. The data were submitted to ANOVA, and the mean values were compared by using the *Fisher* test ($P < 0.05$). The corresponding IC_{50} values were calculated using MINITAB Release 14 statistical software for Windows.

Supplementary Material

Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbdv.201600426.

Acknowledgements

This research was supported by grants from *Consejo de Investigaciones de la Universidad Nacional de*

Tucumán (CIUNT), *Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT)* and *Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET)*. We thanks Dr. *O. Baino*, from Facultad de Agronomía y Zootecnia, UNT, for providing fungi strains and Dr. *N. Muruaga* from Fundación Miguel Lillo, for taxonomic determination of plant material.

References

[1] J. J. Hoffmann, B. E. Kingsolver, S. P. McLaughlin, B. N. Timmermann, *Rec. Adv. Phytochem.* **1984**, *18*, 251.
[2] L. A. Del Vitto, E. M. Petenatti, M. E. Petenatti, *Multequina* **1997**, *6*, 49.
[3] L. Zhou, E. R. Fuentes, J. J. Hoffmann, B. N. Timmermann, *Phytochemistry* **1995**, *40*, 1201.
[4] X. E. Hernández, A. A. Orden, O. S. Giordano, M. Kurina, *Electron. J. Biotechnol.* **2005**, *8*, 277.
[5] S. C. Lin, R. J. Chein, *J. Org. Chem.* **2016**, just accepted manuscript, http://pubs.acs.org/doi/pdfplus/10.1021/acs.joc.6b02766.
[6] D. Wassner, D. Ravetta, *Ind. Crops Prod.* **2000**, *11*, 7.
[7] J. A. Zavala, D. A. Ravetta, *Plant Ecol.* **2002**, *161*, 185.
[8] J. J. Hoffman, B. E. Kingsolver, S. P. McLaughlin, B. N. Timmermann, 'Production of resins by arid-adapted Asteraceae', in 'Phytochemical adaptation to stress', Eds. B. N. Timmermann, C. Steelink, F. Loewus, Plenum Publishing Co., New York, NY, 1984, p. 251.
[9] F. Bohlmann, M. Ahmed, N. Borthakur, M. Wallmeyer, J. Jakupovic, R. M. King, H. Robinson, *Phytochemistry* **1982**, *21*, 167.
[10] B. N. Timmermann, D. J. Luzbetak, J. J. Hoffmann, S. D. Jolad, K. H. Schram, R. B. Bates, R. E. Klenck, *Phytochemistry* **1983**, *22*, 523.
[11] B. Williamson, B. Tudzynski, P. Tudzynski, J. A. van Kan, *Mol. Plant Pathol.* **2007**, *8*, 561.
[12] M. Joshi, S. D. Kamat, D. V. Kamat, *Bionano. Frontier* **2014**, *7*, 34.
[13] M. L. Arias Cassará, S. A. Borkosky, M. González Sierra, A. Bardón, M. I. Ybarra, *Chem. Biodiversity* **2010**, *7*, 1745.
[14] E. Guerreiro, J. Kavka, J. R. Saad, M. A. Oriental, O. S. Giordano, *Rev. Latinoam. Quím.* **1981**, *12*, 77.
[15] B. N. Timmermann, J. J. Hoffmann, S. D. Jolad, K. H. Schram, *Phytochemistry* **1985**, *24*, 1031.
[16] B. N. Timmermann, J. J. Hoffmann, S. D. Jolad, R. B. Bates, T. J. Siahaan, *Phytochemistry* **1987**, *26*, 467.
[17] A. A. Mahmoud, A. A. Ahmed, T. Tanaka, M. Inuma, *J. Nat. Prod.* **2000**, *63*, 378.
[18] A. A. Ahmed, A. A. Mahmoud, U. M. Ahmed, A. A. El-Bassuony, M. H. El-Rask, P. W. Pare, J. Kaechesy, *J. Nat. Prod.* **2001**, *64*, 1365.
[19] D. Holme, N. A. Sorensen, *Acta Chem. Scand.* **1954**, *8*, 34.
[20] J. Jakupovic, R. N. Baruah, C. Zdero, F. Eid, V. P. Eid, V. P. Pathak, V. Chau-Thi, F. Bohlmann, R. M. King, H. Robinson, *Phytochemistry* **1986**, *25*, 1873.
[21] A. F. Rose, K. C. Jones, W. F. Haddon, D. L. Dreyer, *Phytochemistry* **1981**, *20*, 2249.
[22] L. Mangoni, M. Belardini, *Gazz. Chim. Ital.* **1962**, *92*, 983.
[23] P. J. Hoffmann, S. D. Jolad, B. N. Timmermann, R. B. Bates, F. A. Camou, *Phytochemistry* **1988**, *27*, 493.
[24] B. Viswanath, B. Rajesh, A. Janardhan, A. P. Kumar, G. Narasimha, *Enzyme Res.* **2014**, http://dx.doi.org/10.1155/2014/163242.
[25] W. Kopeć, J. Telenius, H. Khandelia, *FEBS J.* **2013**, *280*, 2785.
[26] M. Gilabert, A. N. Ramos, M. M. Schiavone, M. E. Arena, A. Bardón, *J. Nat. Prod.* **2011**, *74*, 574.
[27] S. Amaya, J. A. Pereira, S. A. Borkosky, J. C. Valdez, A. Bardón, M. E. Arena, *Phytomedicine* **2012**, *19*, 173.
[28] J. Gershenzon, N. Dudareva, *Nat. Chem. Biol.* **2007**, *3*, 408.
[29] J. H. Tatum, R. A. Baker, R. E. Berry, *Phytochemistry* **1985**, *24*, 457.
[30] J. M. Sánchez-Calvo, G. R. Barbero, G. Guerrero-Vásquez, A. G. Durán, M. Macías, M. A. Rodríguez-Iglesias, J. M. G. Molinillo, F. A. Macías, *Med. Chem. Res.* **2016**, *25*, 1274.
[31] J. A. Valderrama, H. Leiva, J. A. Rodriguez, C. Theoduloz, G. Schmeda-Hirshmann, *Bioorg. Med. Chem.* **2008**, *16*, 3687.
[32] U. Rohnert, I. Heiser, S. Nemec, R. Baker, W. Osswald, E. F. Elstner, *J. Plant Physiol.* **1998**, *153*, 684.
[33] M. González-Sierra, A. C. Olivieri, M. I. Colombo, E. A. Rúveda, *J. Chem. Soc., Perkin Trans.* **1989**, *1*, 1393.
[34] M. González Sierra, M. I. Colombo, A. C. Olivieri, M. E. Zudenigo, E. A. Ruveda, *J. Org. Chem.* **1984**, *49*, 4984.
[35] J. Hoffmann, S. P. McLaughlin, S. D. Jolad, K. Schram, M. S. Tempesta, R. B. Bates, *J. Org. Chem.* **1982**, *47*, 1725.
[36] A. F. Rose, *Phytochemistry* **1980**, *19*, 2689.
[37] A. C. Olivieri, M. González-Sierra, E. A. Rúveda, *J. Org. Chem.* **1986**, *51*, 2824.
[38] M. González-Sierra, M. I. Colombo, M. E. Zudenigo, E. A. Rúveda, *Phytochemistry* **1984**, *23*, 1685.

Received November 5, 2016
Accepted January 20, 2017

