Phytotoxic Eremophilane Sesquiterpenes from the Coprophilous Fungus *Penicillium* sp. G1-a14

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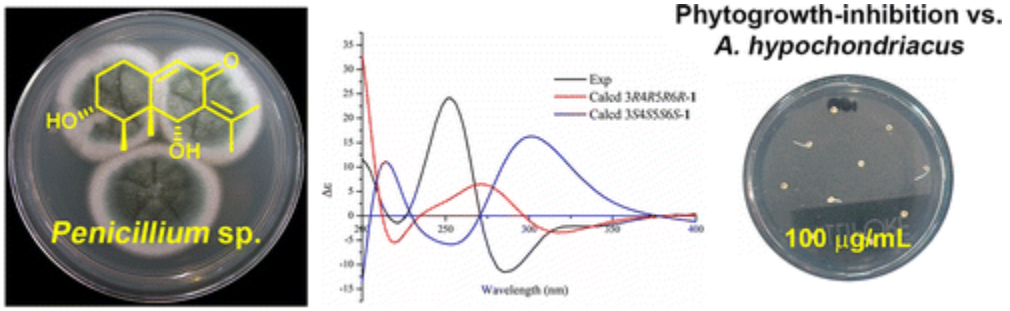
Abstract:

Bioassay-directed fractionation of an extract from the grain-based culture of the coprophilous fungus *Penicillium* sp. G1-a14 led to the isolation of a new eremophilane-type sesquiterpene, 3*R*,6*R*-dihydroxy-9,7(11)-dien-8-oxoeremophilane (**1**), along with three known analogues, namely, isopetasol (**2**), sporogen AO-1 (**3**), and dihydrosporogen AO-1 (**4**). The structure of **1** was elucidated using 1D and 2D NMR and single-crystal X-ray diffraction. Assignment of absolute configuration at the stereogenic centers of **1** was achieved using ECD spectroscopy combined with time-dependent density functional theory calculations. Sporogen AO-1 (**3**) and dihydrosporogen AO-1 (**4**) caused significant inhibition of radicle growth against *Amaranthus hypochondriacus* (IC_{50} = 0.17 mM for both compounds) and *Echinochloa crus-galli* (IC_{50} = 0.17 and 0.30 mM, respectively).



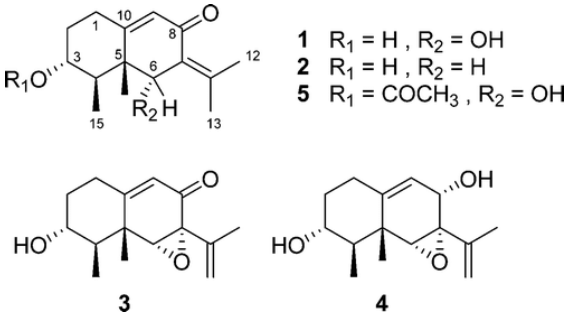
Keywords: *Penicillium* | herbicides | weed controls | ascomycete

Article:

Weed control relies mainly on synthetic herbicides, although these are highly toxic to many living organisms and cause significant environmental pollution.(1, 2) In this regard, the agrochemical industry is continuously looking for the development of new natural pesticides that are environmentally more friendly.(3) Coprophilous fungi have been an important source of bioactive secondary metabolites, including antifungal, antibacterial, and herbicidal agents.(4, 5)

As part of our systematic search for potential herbicidal agents, we have now investigated *Penicillium* sp. G1-a14, an ascomycete isolated from bat guano collected in the Chontalcoatlán cave located in the State of Guerrero, Mexico.

Penicillium sp. G1-a14 was selected for bioassay-directed fractionation on the basis of its phytogrowth-inhibitory activity (Figure S13, Supporting Information) against seedlings of *Amaranthus hypochondriacus* L. and *Echinochloa crus-galli* (L) Beauv., commonly known as amaranth and barnyard grass, respectively. Extensive chromatography of the active extract led to the isolation of a new eremophilane sesquiterpene (**1**), along with three known analogues, namely, isopetasol (**2**), sporogen AO-1 (**3**), and dihydrosporogen AO-1 (**4**). Eremophilanes **2**–**4** were identified by comparison with spectroscopic data previously reported (Figures S7–S12).(6-8)



Compound **1** was isolated as a colorless, crystalline, and optically active compound. HRESIMS data gave a molecular formula of $C_{15}H_{22}O_3$. The ^{1}H and ^{13}C NMR spectra (Figures S1 and S2 and Table 1) revealed that **1** was the deacetyl analogue of 3-acetyl-9,7(11)-diene-6α-hydroxy-8-oxoeremophilane (**5**).(9) The main differences between the spectra of these compounds were the absence of the resonances for the 3-acetyl moiety and the chemical shift values of H-3/C-3 (δ_H/δ_C 3.56/72.0), which appeared diamagnetically shifted in **1**. The structure of eremophilane **1** was corroborated by the key correlations observed in the 2D NMR experiments (Figures S3–S6 and Table 1) and an X-ray crystallographic analysis. As depicted in Figure 1, the cyclohexenone ring in compound **1** adopted an envelope-like conformation, while the second cyclohexane ring displayed a chair conformation. The hydroxy groups are oriented in the same direction. On the basis of the X-ray structure and the NOESY experiment, the absolute configuration at the stereogenic centers of **1** could be either 3*R*,4*R*,5*R*,6*R* or 3*S*,4*S*,5*S*,6*S.* In order to discriminate between these two possibilities, the ECD spectrum of **1** was recorded and compared with those calculated for each enantiomer using the time-dependent density functional theory (TDDFT) method. Conformational analysis of the two possible stereoisomers, 3*R*,4*R*,5*R*,6*R* and 3*S*,4*S*,5*S*,6*S*, was undertaken using the Monte Carlo protocol. All conformers for each enantiomer, within a 4 kcal/mol energy window, were selected and optimized using DFT calculations at the B3LYP/DGDZVP level. After optimization, the theoretical ECD spectrum of each enantiomer was calculated using TDDFT at the same level (Tables S1 and S2).(10-12) The calculated ECD spectrum for enantiomer 3*R*,4*R*,5*R*,6*R* showed a good fit with the experimental plot of **1**, which displayed two negative and one positive Cotton effects at 218, 318, and 272 nm, respectively (Figure 2). On the other hand, the calculated ECD spectrum of the 3*S*,4*S*,5*S*,6*S* enantiomer was opposite to the experimental ECD data (Figure 2), with two positive and one negative Cotton effect at 214, 302, and 254 nm, respectively. Hence, the 3*R*,4*R*,5*R*,6*R*

configuration of **1** was confirmed. On the basis of the above discussion compound **1** was identified as 3*R*,6*R*-dihydroxy-9,7(11)-dien-8-oxoeremophilane.

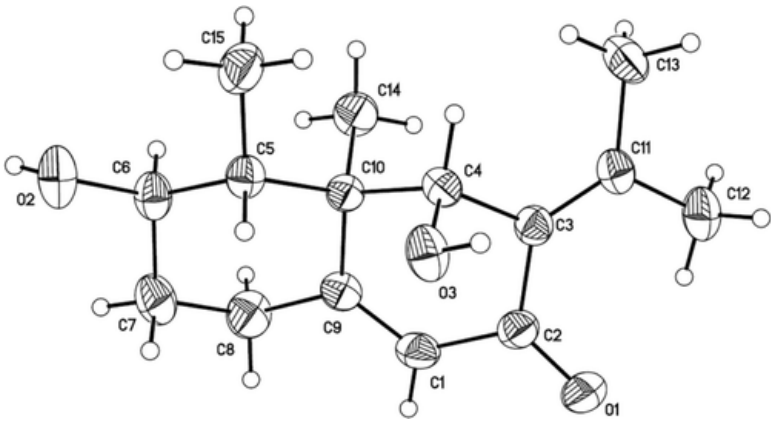


Figure 1. ORTEP drawing of compound **1** showing atomic labeling.

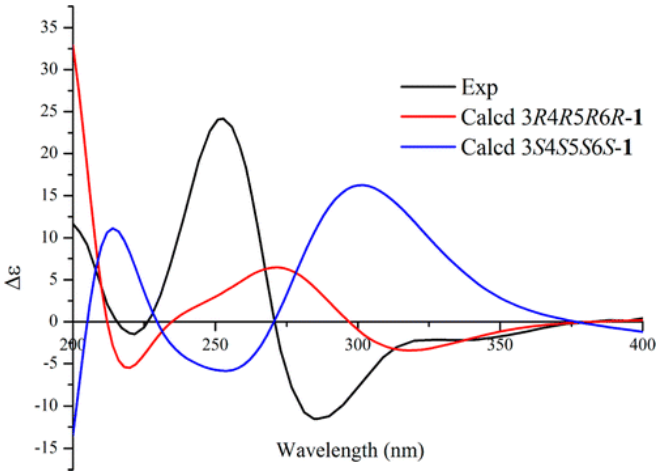


Figure 2. Comparison of experimental ECD spectrum of **1** (black) with those calculated at the B3LYP/DGDZVP level for enantiomers 3*R*,4*R*,5*R*,6*R* (red) and 3*S*,4*S*,5*S*,6*S* (blue).

Table 1. NMR Spectroscopic Data of Compound 1 (CD_3OD)[a]

position	δ_C, type[b]	δ_H, (mult, *J* in Hz)[c]	HMBC	NOESY
1	31.8, CH_2	2.41, m 2.50, m	2, 5, 3, 9, 10	2, 3, 9, 14
2	35.5, CH_2	2.05, m 1.45, m	1, 3, 4, 10	1, 3
3	72.0, CH	3.56, td (4.1, 10.9)	1, 2, 4, 15	1, 2, 14, 15
4	41.7, CH	2.14, m	2, 3, 5, 6, 14, 15	2, 15
5	47.1, C			
6	73.0, CH	4.70, s	1, 4, 5, 7, 8, 11, 14	13, 14, 15
7	133.3, C			
8	193.2, C			
9	125.8, CH	5.74, d (1.6)	1, 5, 7, 11	1, 14
10	166.0, C			
11	147.0, C			
12	23.0, CH_3	2.07, s	6, 7, 8, 11, 13	
13	21.9, CH_3	1.95, s	7, 8, 11, 12	6

position	δ_C, type[b]	δ_H, (mult, *J* in Hz)[c]	HMBC	NOESY
14	18.0, CH_3	0.98, s	4, 5, 6, 10, 11	1, 3, 6, 13, 15
15	10.4, CH_3	1.10, d (6.7)	3, 4, 5	4, 3, 6

[a] Chemical shifts (δ) in ppm.
[b] 100 MHz.
[c] 400 MHz.

The phytogrowth-inhibitory assay(13) for sporogen AO-1 (**3**) and dihydrosporogen AO-1 (**4**) revealed a concentration-dependent inhibition on radicle elongation against *A. hypochondriacus and E. crus-galli*. Table 2 summarizes the IC_{50} values for the extract of *Penicillium* sp. G1-a14 and the isolated eremophilanes sporogen AO-1 (**3**) and dihydrosporogen AO-1 (**4**). Compound **1** did not exhibit phytogrowth-inhibitory activity up to a concentration of 1.0 mM. Isopetasol (**2**) could not be tested due to the scarcity of sample.

Table 2. Phytogrowth-Inhibitory Activity (IC_{50}) of the Organic Extract and Compounds 3 and 4 from *Penicillium* sp. G1-a14 against *A. hypochondriacus* and *E. crus-galli*

compound	*Amaranthus hypochondriacus*	*Echinochloa crus-galli*
extract[a]	46.18	184.74
3[b]	0.17	0.17
4[b]	0.17	0.30
tricolorin A[b,c]	0.10	0.03

[a] Expressed in μg/mL.
[b] Expressed in mM.
[c] Positive control.

In summary, one new and two bioactive eremophilanes were isolated from the coprophilous fungus *Penicillium* sp. G1-a14. This type of sesquiterpenoid is common in *Penicillium*, *Aspergillus*, and *Xylaria* species.(14)

Experimental Section

General Experimental Procedures

Melting points were determined on a Fisher-Johns apparatus and are uncorrected. IR, UV, and ECD spectra were obtained on a PerkinElmer 400 FT-IR, a Shimadzu U160, and a JASCO model J720 spectrophotometer, respectively. Optical rotations were recorded at the sodium D-line wavelength using a PerkinElmer model 343 polarimeter at 20 °C. 1D and 2D NMR spectra were recorded on a 400 MHz Bruker Avance III (operating at 400.13 MHz for ^1H and 100.61 MHz for ^{13}C; in the case of **1**), a Varian Inova 300 MHz (operating at 300.13 MHz for ^1H and 75.15 MHz for ^{13}C; in the case of **4**), or a Varian Unity Inova 500 MHz (operating at 500 MHz for ^1H and 125.71 MHz for ^{13}C; in the case of **2** and **3**) spectrometer; spectra were recorded using $CDCl_3$ or CD_3OD and tetramethylsilane (TMS) as an internal standard. HRESIMS spectra were obtained using a Thermo LTQ Orbitrap XL mass spectrometer. HPLC was carried out on a Waters system equipped with a 2535 pump and a 2998 photodiode array detector; data acquisition and management of chromatographic output were performed with the Empower 3 software (Waters). Reagent grade dichloromethane, *n*-hexane, methanol, and HPLC grade acetonitrile and methanol (J.T. Baker) were regularly used in the extraction and isolation procedures. Silica gel 60 (70–230 mesh, Merck) and Sephadex LH-20 (General Electric) were used for column chromatography (CC). TLC analyses were performed on precoated silica gel 60

F_{254} plates (Merck) using different mobile phases; visualization of plates was carried out using a $Ce_2(SO_4)_3$ (10%) solution in H_2SO_4 and heating.

Isolation and Identification of *Penicillium* sp. G1-a14

Samples of fresh bat guano were collected aseptically from two locations throughout the Chontalcoatlán cave in the State of Guerrero, México (18°38′52″ N, 99°31′4″ W) in February 2013, using sterile glass tubes and a spatula. The samples were vortexed with 3 mL of sterile water; 0.5 mL of the supernatants was directly plated onto water–agar medium (consisting of 39 g of agar in 1 L of distilled water) and potato-dextrose agar (PDA, Difco). Both media were amended with streptomycin sulfate (30 mg/L) and rose bengal (50 mg/L). Agar plates were incubated aerobically at room temperature for 1 week and screened for the presence of single fungal colonies. Colonies were transferred and purified on new PDA medium. One of these isolated colonies was accessioned as G1-a14 and is preserved in the Department of Pharmacy culture collection at Facultad de Química, Universidad Nacional Autónoma de México, México.

The macromorphology and micromorphology of the fungus allowed us to preliminarily assign G1-a14 as *Penicillium* sp. (Trichocomaceae, Eurotiales, Eurotiomycetideae, Eurotiomycetes, Ascomycota).(15) For molecular identification of G1-a14, DNA was extracted from fresh cultures grown on PDA. The nuclear internal transcribed spacer regions 1 and 2 and 5.8S nrDNA (ITS) were amplified and sequenced following published protocols.(15) The consensus sequence of the ITS region was submitted for BLAST search using the NCBI GenBank database, and the top BLAST matches for G1-a14 indicated similarities to sequences of several *Penicillium* spp. [*P. copticola* (GenBank JN617685, identities = 364/386, 94%), *P. terrigenum* (GenBank JN617684, identities = 364/386, 94%), *P. roseopurpureum* (GenBank AF455437, identities = 364/386, 94%), and *P. sanguifluum* (GenBank JN617689, identities = 359/386, 93%)]. On the basis of this information, the fungus is currently recognized as *Penicillium* sp. G1-a14, and the ITS sequence was deposited in GenBank (accession no. KP402588).

Fermentation, Extraction, and Isolation

A 15 mL amount of seed cultures grown for 1 week in potato-dextrose broth (Difco) was used to inoculate two Fernbach flasks containing the solid rice medium (250 g of rice and twice the volume of rice with H_2O, sterilized by autoclaving at 121 °C for 15 min). Fermentation was carried out at room temperature until good growth was observed (approximately 30 days). The solid cultures were extracted three times with 500 mL of CH_2Cl_2–MeOH (9:1). The cultures were chopped with a spatula and shaken overnight at ∼100 rpm, followed by vacuum filtration. The filtrates were transferred into a separatory funnel. The bottom layers were combined and dried over anhydrous Na_2SO_4, then evaporated to dryness to produce 3 g of a brownish residue, which was subsequently reconstituted with 100 mL of MeOH–CH_3CN (1:1) and 100 mL of *n*-hexane. The biphasic solution was transferred to a separatory funnel and shaken vigorously. The MeOH–CH_3CN layer was drawn off and evaporated to dryness under vacuum to yield 1.5 g of a defatted fraction. One gram of the latter was subjected to Sephadex LH-20 CC eluting with MeOH–CH_2Cl_2 (9:1) to yield five major secondary fractions (F1–5). F4 (350 mg) was further separated by CC on silica gel eluting with a gradient of *n*-hexane–CH_2Cl_2 (100:0 → 0:100) and CH_2Cl_2–MeOH (100:0 → 70:30) to generate 10 fractions ($F4_{I–X}$). Subfractions $F4_{VII}$ and

$F4_{IX}$ yielded compounds **4** (15.8 mg) and **1** (18.8 mg), respectively. Eremophilanes **2** (4.3 mg) and **3** (10.2 mg), which eluted at 10.5 and 13.5 min, respectively, were purified from $F4_V$ (45 mg) by reversed-phase HPLC (Gemini C_{18}, 250 × 21.2 mm, 5 μm) using as mobile phase MeOH–H_2O (acidified with 0.1% formic acid), 1:1, and increasing linearly to 80% MeOH over 30 min, then changing to 100% MeOH for 5 min. The flow rate was 18 mL/min, and the injection volume was 300 μL.

3*R*,6*R*-Dihydroxy-9,7(11)-dien-8-oxoeremophilane (1):

colorless needles ($CHCl_3$); mp 206 °C; $[\alpha]^{20}_D$ −13.68 (*c* 1.9, H_2O); UV (H_2O) λ_{max} (log ε) 255.2 (4.2) nm; IR (ATR) ν_{max} 1650 and 3500 cm^{-1}; ^1H NMR and ^{13}C NMR see Table 1; ESIMS *m*/*z* 273 $[M + Na]^+$ (82), 251 (61), 233 (65), 215 (100), 188 (5), 161 (4), 122 (5); HRESIMS *m*/*z* 251.1646 $[M + H]^+$ (calcd for $C_{15}H_{23}O_3$, 251.1642).

X-ray Crystal Structure Analysis of Compound 1

X-ray diffraction data were collected on a Bruker D8 Venture diffractometer with Cu Kα radiation. The structure was solved by the SHELXS-2013 method and refined using full-matrix least-squares on F^2. Suitable crystals of **1** were obtained by evaporation of CH_2Cl_2–MeOH (9:1). Crystallographic data for **1** have been deposited with the Cambridge Crystallographic Data Centre (CCDC) with the accession no. 1034405. These data are available, free of charge, from the CCDC via http://www.ccdc.cam.ac.uk/data_request/cif.

Crystal data for 1

$C_{15}H_{22}O_3$, M = 250.32, orthorhombic, space group $P2_12_12_1$ with a = 10.8914(2) Å, b = 10.9342(2) Å, c = 11.4227(2) Å, α = β = γ = 90°, V = 1360.31(4) $Å^3$, Z = 4, T = 296(2) K, D_c = 1.222 Mg/m^3, μ(Cu Kα) = 0.669 mm^{-1}, F(000) = 544, 27 500 reflections collected, 2481 independent reflections (R_{int} = 0.0459). The final R_1 values were 0.0329 [$I > 2\sigma(I)$]. The final wR_2 (F^2) values were 0.0868 [$I > 2\sigma(I)$]. The final R_1 values were 0.0331 (all data). The final wR_2 (F^2) values were 0.0870 (all data).

Computational Section

Minimum energy structures for the different stereoisomers were built with Spartan'10 software (Wavefunction Inc.). Conformational analysis was performed with the Monte Carlo search protocol as implemented in the same software under the MMFF94 molecular mechanics force field. The resulting conformers were minimized using the DFT method at the B3LYP/DGDZVP level of theory employing the Gaussian'09 program package (Gaussian Inc.). The self-consistent reaction field with conductor-like continuum solvent model was used to perform the ECD calculations of the major conformers of both **1** enantiomers in water solution. The calculated excitation energy (nm) and rotatory strength (R) in dipole velocity (R_{vel}) and dipole length (R_{len}) forms were simulated into an ECD curve.

Phytogrowth-Inhibitory Bioautographic Assay

Seeds of *Amaranthus hypochondriacus* L. and *Echinochloa crus-galli* L. Beauv. were first pretreated with sodium hypochlorite (5%) for 1 min and washed exhaustively with distilled water. Then, TLC was performed on precoated silica gel 60 F_{254} glass plates (3 × 20 cm) for extract and fractions (1 mg, Figure S13). After eluting with CH_2Cl_2–MeOH (95:5), plates were dried and covered with 15 mL of agar solution (1%). When the agar was solidified, seeds were placed over the plate area (~15 seeds per cm^2). Phytogrowth-inhibition bands were observed after incubation of 36 h for *A. hypochondriacus* or 48 h for *E. crus-galli*, at 28 °C in a moistened chamber.

Phytogrowth-Inhibitory Biossay

The phytotoxic activity of the extract and isolated metabolites of *Penicillium* sp. G1-a14 was evaluated on radicle growth of *A. hypochondriacus* and *E. crus-galli* using a Petri dish bioassay.(16) Seeds were pretreated as described above before germination. Stock solutions of the extract and compounds in MeOH were used to prepare agar solutions (2%) of the extract (50, 100, 150, and 200 μg/mL) and pure compounds (25, 50, 75, and 100 μg/mL). A 5 mL sample of varying concentrations of agar solution was added to each Petri dish. After agar solidification, 10 seeds were placed on each plate. Three replicates were prepared for each concentration; tricolorin A was used as the positive control.(13) Dishes were kept in a moistened chamber at 28 °C. After 36 or 48 h, for *A. hypochondriacus* and *E. crus-galli*, respectively, the root length was measured and compared to the proper untreated control (MeOH). The inhibition percent was calculated using the formula $\text{Inhibition (\%)} = (L_{\text{control}} - L_{\text{treatment}})/L_{\text{control}} \times 100$ where L_{control} is the radicle length of seedlings in the control and $L_{\text{treatment}}$ is the radicle length of seedlings treated. The IC_{50} values were calculated by linear fitting based on percent of radicle growth inhibition.

Supporting Information

1D and 2D NMR spectra of compounds **1**–**4**, X-ray crystallographic data of **1**, calculated DFT B3LYP/DGDZVP free energies, population and theoretical averaged rotatory strength values expressed in $R_{(len)}$ for conformers of 3*R*4*R*5*R*6*R* and 3*S*4*S*5*S*6*S* enantiomers of **1**, and phytogrowth-inhibitory activity (bioautographic assay) of active fraction F4 are available free of charge via the Internet at http://pubs.acs.org.

Notes

† Taken in part from the Ph.D. thesis of P. Del Valle.

The authors declare no competing financial interest.

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