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Phytochemistry 66 (2005) 1012–1016
PHYTOCHEMISTRY
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Phytotoxins from the fungus *Malbranchea aurantiaca* ☆

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Received 10 December 2004; received in revised form 8 February 2005
Available online 16 April 2005

Abstract

Bioassay-directed fractionation of an ethyl acetate extract from cultures of the fungus *Malbranchea aurantiaca* led to the isolation of two phytotoxic compounds, namely, 1-hydroxy-2-oxoeremophil-1(10),7(11),8(9)-trien-12(8)-olide (**1**) and penicillic acid (**2**). The structure of **1** was established by spectroscopic and X-ray crystallographic analyses. Metabolites **1** and **2** caused significant inhibition of radicle growth of *Amaranthus hypochondriacus* with IC_{50} values 6.57 and 3.86 μM, respectively. In addition, **1** inhibited activation of the calmodulin-dependent enzyme *c*AMP phosphodiesterase (IC_{50} = 10.2 μM).

Keywords: *Malbranchea aurantiaca*; *Amaranthus hypochondriacus*; Eremophilane; Sesquiterpenoids; 1-Hydroxy-2-oxoeremophil-1(10),7(11),8(9)-trien-12(8)-olide; Calmodulin; *c*AMP phosphodiesterase; Phytotoxins

1. Introduction

Continuing our research on bioactive agents from fungi, we have investigated *Malbranchea aurantiaca* Sigler & Carmich (Myxotrichaceae) (Macías et al., 2000, 2001; Rivero-Cruz et al., 2000, 2003), an ascomycete isolated from bat guano. This species has not been previously investigated, but studies of the related species *Malbranchea pullchella* and *Malbranchea cinnamomea* resulted in the isolation of benzoisocumarin and benzoquinone metabolites (Saito et al., 1979; Chiung et al., 1993; Schlegel et al., 2003). *M. aurantiaca* was selected for bioassay-guided fractionation in the present investigation on the basis of its phytogrowth inhibitory activity against *Amaranthus hypochondriacus* (IC_{50} = 195 μg/ml). Herein, we describe the isolation, structure elucidation and biological activity of the major phytotoxin from *M. aurantiaca,* including its effect on the regulatory protein calmodulin (CaM) as a possible target of phytotoxic action.

2. Results and discussion

The active (IC_{50} = 195.0 μg/ml against *A. hypochondriacus*) organic extract (mycelial and culture broth) of *M. aurantiaca*, isolated from bat guano in a Juxtlahuaca Cave, Mexico, was crudely fractionated by open column chromatography, and the fractions were submitted for phytotoxicity testing. Three fractions (FI, FM and FN) showed radicle growth inhibition in a bioautographic bioassay. Further chromatographic separation of the active fractions yielded 1-hydroxy-2-oxoeremophil-1(10),7(11),8(9)-trien-12(8)-olide (**1**), a novel natural product, and penicillic acid (**2**) (Keromnes and Thouvenor, 1985).

☆ Taken in part from the PhD thesis of Sergio Martínez-Luis.

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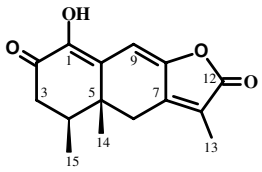
doi:10.1016/j.phytochem.2005.03.005



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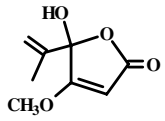

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Compound **1** was isolated as a yellow crystalline, optically active compound. HREIMS data for **1** gave a molecular formula of $C_{15}H_{16}O_4$ with an inherent eight degrees of unsaturation. The IR spectrum was consistent with the presence of conjugated carbonyl functionalities including a γ-lactone (1779 cm^{-1}) and a conjugated ketone (1665 cm^{-1}). The ^{13}C NMR spectrum for **1** (Table 1) revealed 15 resonances, interpreted from the multiplicity-edited HSQC data as eight non-protonated, two methine, two methylene, and three methyl carbons. In the ^{1}H NMR spectrum of **1** (Table 1), its conjugated unsaturated nature was immediately evident from the one olefinic proton resonance (H-9) accompanied by two deshielded methylene resonances (H-3 and H-6). This spectrum also exhibited resonances for three methyl groups (H-13–H-15) and one hydroxyl functionality. Five of the eight degrees of unsaturation were accounted for by the two carbonyl (C-2 and C-12) and six olefinic carbon resonances (C-1 and C-7–C-11) in the ^{13}C NMR spectrum. Therefore, the remaining three degrees of unsaturation were attributed to three carbocyclic rings. Altogether, this information revealed that **1** was an eremophilane sesquiterpene, similar to (−) ligularenolide (Jenniskens and Groot, 1998; Ishizaki et al., 1970) and PF1092 A-C (Tabata et al., 1997), i.e., possessing a conjugated methyl γ-lactone between C-7 and C-8, an α–β unsaturated ketone group at C-2, and a trisubstituted conjugated double bond between C-8 and C-9 and one hydroxyl at C-1. The positions of the functional groups along the eremophilane core were established by an HMBC experiment (Table 1). In particular, the correlations C-10/OH-1, H-6, H-9; C-8/H-9, H-6, H-13; C-2/OH-1, H-6 b and C-12/H-13 were consistent with the disposition of the ketone, hydroxyl and lactone functionalities. On the basis of this evidence, the structure of **1** was assigned as 1-hydroxy-2-oxoeremophil-1(10),7(11),8(9)-trien-12(8)-olide. This was further corroborated by an X-ray crystallographic analysis (Fig. 1). As depicted in Fig. 1, the cyclohexenone ring in compound **1** adopted an envelope conformation while the second cyclohexene ring displayed a boat-like conformation. The hydroxyl groups are oriented in the same direction and the eremophillane core seems to be stabilized by an intramolecular hydrogen bond between the hydroxyl group [O-5 (O-1)] and the carbonyl groups [O-6 (O-2)] on both molecules (Fig. 2). Finally, the absolute configuration of compound **1** was proposed as depicted on the basis of the CD spectrum which displayed a strong negative Cotton effect at 378 nm (Kingston et al., 1975; Legrand and Rougier, 1977). Furthermore, like (−) ligularenolide (Jenniskens and Groot, 1998), whose absolute stereochemistry was determined by chemical correlation, compound **1** possesses a negative optical rotation.

Compounds **1** and **2** showed phytotoxic properties when tested against seedlings of *A. hypochondriacus* using a petri dish bioassay. Compounds **1** [IC_{50} = 6.57 μM) and **2** [IC_{50} = 3.86 μM) inhibited radicle growth of this species with a similar potency to 2,2-dichlorophenoxyacetic acid [2,4-D; IC_{50} = 18 μM], which was used as a positive control. The phytotoxic effect of **1** was also comparable to other eremophilanes such as dehydroflourensic acid, previously isolated in our group from the plant species *Flourensia cernua* (Mata et al., 2003). Keromnes and Thouvenor (1985) showed that compound **2** was highly toxic to corn seeds.

Table 1
^{1}H (500 MHz) and ^{13}C (125 MHz) NMR ($CDCl_3$) spectroscopic data for 1-hydroxy-2-oxoeremophil-1(10),7(11),8(9)-trien-12(8)-olide

Position	$^{13}C(\delta)$	^{1}H (δ, *m*, *J* in (Hz))	HMBC (C → H)
1	144.2		H-3a, OH-1, H-9
2	192.1		H-3a, H-3b, H-15, OH-1
3	40.1	a, 2.56 (*dd* 18, 4.5) b, 2.47 (*dd* 18, 7)	H-15
4	38.5	2.26 (*ddq* 13.5, 4.5, 7)	H-3b, H-6b, H-14, H-15,
5	38.2		H-3a, H-6a, H-4, H-14, H-15, OH-1
6	34.5	a, 2.90 (*dd* 16.5, 0.5) b, 2.41 (*dq* 16.5, 2)	H-4, H-14, H-13
7	145.1		H-6a, H-6b, H-9, H-13
8	150.9		H-6a, H-9, H-13
9	102.3	6.58 (*d* 0.5) H-13	
10	131.6		H-6a, H-14, OH-1
11	123.4		H-6a, H-6b, H-13
12	170.4		H-13
13	8.8	1.98 (*brd* 2.5)	
14	18.7	1.21 (*d* 1.0)	H-4, H-6a, H-6b
15	14.9	1.01 (*d* 6.5)	H-3a, H-3b, H-6b, H-4
OH-1		6.45 (*s*)	

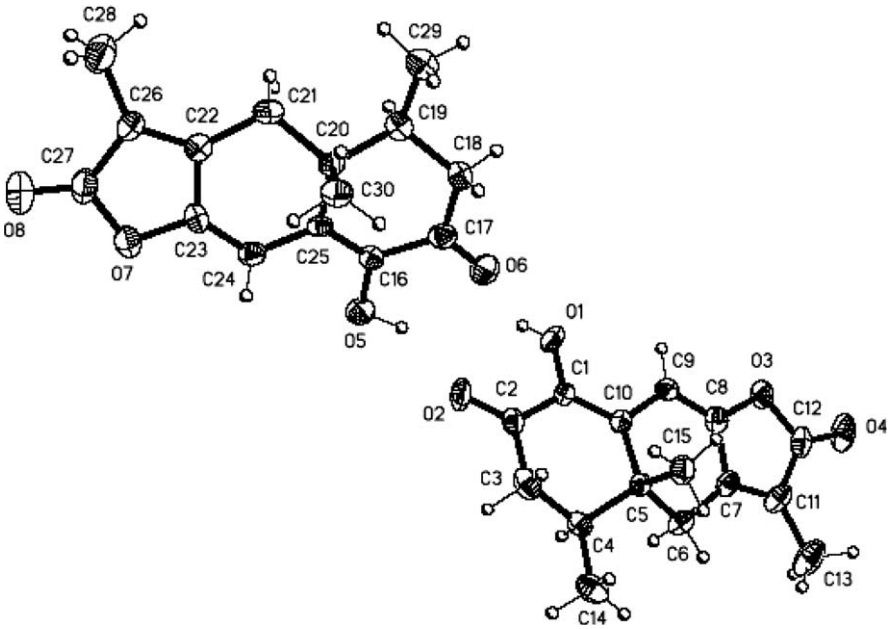

Fig. 1. ORTEP drawing of compound **1**.

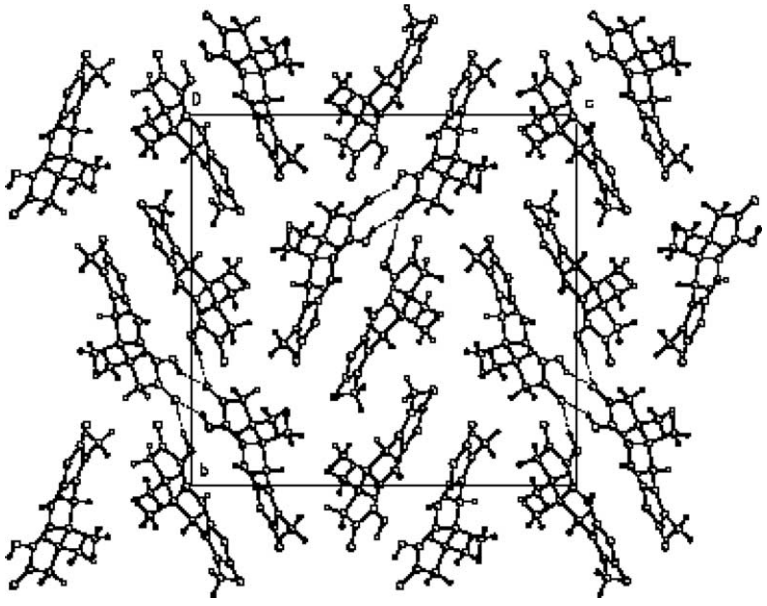

Fig. 2. Unit cell viewed down the *c*-axis for compound **1**.

Although this compound is produced in high quantities in cultures of *Penicillum cyclopium* and *Penicillum canescens* isolated from corn seeds, its toxicity to animals have precluded further development as a herbicidal agent.

The effect of compound **1** on calmodulin (CaM) was also investigated as previously described (Mata et al., 2003). 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 phosphodiesterase, NAD-kinase, protein phosphatase, and nitric oxide (NO) synthase. Accordingly, agents that inhibit the activity of CaM should have profound effects on the development of plants (Snedden and Fromm, 1998; Mattew, 2000; Hook and Means, 2001). Indeed, certain fungal phytotoxins inhibit CaM (Au et al., 2000; Macías et al., 2000, 2001; Rivero-Cruz et al., 2003). The results showed that activation of the calmodulin-sensitive *c*AMP phosphodiesterase was inhibited in the presence of **1** and CaM. The effect of compound **1**($IC_{50} = 10.2 \pm 7.6$ μM) was higher than that of chlorpromazine ($IC_{50} = 18.4 \pm 2.7$ μM), a well known CaM inhibitor used as a positive control.

In conclusion, *M. aurantiaca* biosynthesizes penicillic acid and 1-hydroxy-2-oxoeremophil-1(10),7(11),8(9)-trien-12(8)-olide, a phytotoxin that inhibits the regulatory properties of CaM in vitro. It is important to point out that highly conjugated eremophillanes type of sesquiterpenes, although common in plants, are scarce in fungi; in such organisms they have been found only in the genus *Penicillium* (Tabata et al., 1997), therefore our results could be of chemotaxonomic relevance.

3. Experimental

3.1. General experimental procedures

Melting point determinations were carried out on a Fisher–Johns apparatus and are uncorrected. Optical rotations were recorded on a JASCO DIP 360 digital polarimeter. The CD spectrum of **1** was performed on a JASCO 720 spectropolarimeter at 25 °C in $CHCl_3$ solution. IR spectra were obtained using KBr disks on a Perkin–Elmer 599B spectrophotometer. UV spectra were recorded on a Shimadzu 160 UV spectrometer in CHCl3 solution. NMR spectra including COSY, NOESY, HMBC and HMQC experiments were acquired 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 employed silica gel 60 (70–230 mesh, Merck). TLC (analytical and preparative) was performed on precoated silica gel 60 F_{254} plates (Merck).

3.2. Fungal material

The fungus *M. aurantiaca* Siegler & Carmich was isolated from bat guano collected at the Juxtlahuaca cave located in Ramal del Infierno, State of Guerrero, México, in 1998. A voucher specimen (24428) is deposited in the National Herbarium (MEXU), Instituto de Biología, Nacional Autónoma de México, México City.

Eighteen 2-L Erlenmeyer flasks, each containing 1 L of potato dextrose broth (PDB, Difco), were individually inoculated with 1 cm^2 agar plug taken from a stock culture of *M. aurantiaca* maintained at 4 °C on potato dextrose agar (PDA). Flask cultures were incubated at 28 °C and aerated by agitation on an orbital shaker at 200 rpm during 15 days, in a dark room.

3.3. Extraction and isolation of 1 and 2

After incubation, all flask contents were combined and filtered. The combined culture filtrate (18 L) was extracted exhaustively with EtOAc (3 × 18 L). The combined organic phase was filtered (anhyd. Na_2SO_4) and concentrated in vacuo to give a dark brown solid (2.8 g). The mycelium was also extracted with EtOAc (3 × 2 L). The combined mycelial extract was evapored to yield 1.2 g of a dark brown solid. The extracts (culture and mycelial) were combined (4.0 g) and subjected to Si gel (400 g) open CC eluting with a gradient of hexane–CH_2Cl_2 (10:0 → 0:10) and CH_2Cl_2–MeOH (9.9:0.1 → 1:1) to yield 20 major fractions (FA-FS). Bioactivity in the bioautographic assay showed three active pools: FI (120 mg), FM (20 mg) and FN (25 mg). From the active fraction FI, eluted with hexane–CH_2Cl_2 (7:3), precipitated a yellow crystalline solid which was purified by re-crystallization from MeOH to yield **1** (20 mg). Extensive preparative TLC (CH_2Cl_2) on Si gel of combined active fractions FM and FN yielded **2** (6.0 mg) as the only active compound (Keromnes and Thouvenor, 1985).

3.4. 1-Hydroxy-2-oxoeremophil-1(10),7(11),8(9)-trien-12(8)-olide (1)

Crystalline yellow solid; mp 198–200 °C; $[\alpha]_D$ −187° (CH_2Cl_2; *c* 1); IR ν_{max} (KBr) cm^{-1}: 3357, 1779, 1765, 1665, 1649, 1387, 1371; UV ($CHCl_3$)λ_{max} (logε): 275 (4.18), 378 (4.25) nm; CD $\Delta\varepsilon_{378}$ -6.42×10^4 ($CHCl_3$; *c* 0.1); for ^1H and ^{13}C NMR spectroscopic data, see Table 1; EIMS *m/z* (rel. int.): 260 [M^+ (100)], 245 (18), 232 (45), 217 (25), 203 (7), 189 (13), 175 (7), 161 (7), 147 (6), 91 (6), 69 (10), 41 (7), 18 (19); HRFABMS *m/z* 261.1119 $[M + H]^+$ (calcd for $C_{15}H_{16}O_4$ + H, 261.1127).

3.5. X-ray crystal structure determination of 1

Crystal data of **1**: $C_{15}H_{18}O_4$, MW 260.28, orthorhombic, $P\bar{1}$ with unit cell parameters (at 25 °C) *a* = 6.5856(6) Å, *b* = 19.254(2) Å, *c* = 20.107(2) Å, *V* = 2549.6 (1) $Å^3$, *Z* = 8, *F*(0 0 0) = 1104, D_{calc} = 1.356 mg/m^{-3}, absorption coefficient = 0.098 mm^{-1} (Sheldrick, 1997). A total of 17,367 reflections, of which 4483 were independent, were measured at room temperature from a 0.226 × 0.112 × 0.088 mm yellow prism using a Bruker Smart Apex CCD diffractometer equipped with graphite-monochromated Mo Kα radiation (λ 1.54178 Å).

The structure was determined by direct methods (SIR-92 or SIR-97). All atoms except hydrogens were refined anisotropically by full-matrix least-squares methods on F^2 using SHELXL97 to give a final *R*-factor of 0.0511 (wR_2 = 0.0473) with a data restrains-parameters ratio of 4483/0/355 (Altomare et al., 1994).

Crystallographic data for the structure reported in this paper have been deposited with the Cambridge Crystallograpic Data Centre (CCDC 262766). Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK.

3.6. Phytogrowth-inhibitory bioassays

The phytogrowth-inhibitory activity of the crude extract, fractions and pure compounds was evaluated on seeds of *A. hypochondriacus* using a petri dish bioassay (Macías et al., 2000). In addition, a bioautographic phytogrowth inhibitory bioassay (Macías et al., 2000) was employed to guide secondary fractionation. The seeds of *A. hypochondriacus* were purchased from Mercado de Tulyehualco, Mexico City. 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. Samples were evaluated at 10, 100 and 1000 μg mL^{-1}. 2,4-D was used as the positive control. The bioassays were performed at 28 °C.

3.7. Cyclic nucleotide phosphodiesterase assay

A cyclic nucleotide phosphodiesterase and bovine-brain calmodulin assay was performed as previously described (Rivero-Cruz et al., 2003). Compound **1** was tested at 10, 20, 40, 60, 80, and 100 μM in DMSO. Chlorpromazine was used as a positive control.

Acknowledgements

This work was supported by a Grant from DGAPA-UNAM IN200902. We thank Marisela Gutiérrez, Georgina Duarte-Lisci and Rosa del Villar for recording IR, UV, CD, MS and NMR spectra. Special thanks are due to Laura Acevedo, Isabel Rivero and Samuel Aguilar for their technical assistance. We are also grateful to Veronica Gurrola for her assistance with the PDE-1 assay.

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