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Short Report

New Bioactive Metabolites Produced by *Phomopsis cassiae*, an Endophytic Fungus in *Cassia spectabilis*

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Dois novos metabólitos, 2,4-diidroxi-5,6-dimetil benzoato de etila (**1**) e phomopsilactona (**2**) foram isolados de *Phomopsis cassiae,* um fungo endofítico de *Cassia spectabilis.* As estruturas destes compostos foram elucidadas por dados espectrométricos de 1D e 2D RMN, EM e IV. As substâncias **1** e **2** exibiram forte atividade antifúngica contra os fungos fitopatogênicos *Cladosporium cladosporioides* e *C. sphaerospermum*, bem como citotoxidade contra a linhagem celular de tumor cervical humano (HeLa), em experimentos *in vitro*.

Two new metabolites, ethyl 2,4-dihydroxy-5,6-dimethylbenzoate (**1**) and phomopsilactone (**2**) were isolated from *Phomopsis cassiae*, an endophytic fungus in *Cassia spectabilis*. Their structures were elucidated by 1D and 2D NMR, MS and IR spectral data. Compounds **1** and **2** displayed strong antifungal activity against the phytopatogenic fungi *Cladosporium cladosporioides* and *C. sphaerospermum*, as well as cytotoxicity against human cervical tumor cell line (HeLa), in *in vitro* assays.

Keywords: endophytic fungi, *Phomopsis cassiae*, *Cassia spectabilis*, cytotoxic metabolites, antifungal activity

Introduction

The genus Cassia, comprising about 600 species widely distributed worldwide, is well known for its diverse biological and pharmacological properties.[1] *Cassia spectabilis* (sin *Senna spectabilis*) (DC) Irwin et Barn (Leguminosae) has been used in traditional Brazilian medicine for the treatment of flu and cold, as laxative and purgative.[2] This observation prompted us to launch a program aiming to search novel bioactive metabolites from cultures of endophytes colonized inside *C. spectabilis*. Leaves of *C. spectabilis* were submitted to isolation of endophytic fungi and seven isolates were obtained, preserved and cultivated on liquid medium to get their crude extracts. The strain *Phomopsis cassiae* was selected for chemical and biological investigation because of the strong antifungal activity against the phytopatogenic fungi *Cladosporium sphaerospermum* and *C. cladosporioides*.

Fractionation of the crude EtOAc extract by flash chromatography column on reversed-phase C-18 silica, followed by reversed-phase HPLC, afforded compounds **1-2** and 2-hydroxyphenylacetic acid (**3**). The structures of the new metabolites, ethyl 2,4-dihydroxy-5,6-dimethyl benzoate (**1**) and phomopsilactone (**2**) were determined by analysis of NMR and MS data.

Results and Discussion

Compound **1** was isolated as a white amorphous solid and its molecular formula $C_{11}H_{14}O_4$ was deduced from ^{13}C NMR and ESI-MS data [$(M + H)^+$ at *m/z* 211]. This formula displayed five degrees of unsaturation. The ^1H, ^{13}C NMR

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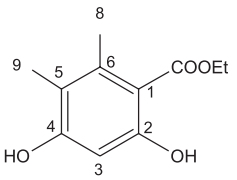
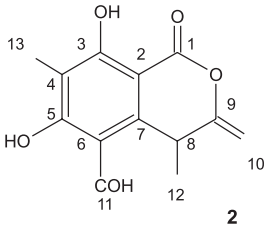
(Table 1) and DEPT results for **1** suggested one phenyl group (five quaternaries carbons), an ester group, a -CH_2CH_3 moiety and two aromatic methyls. The ester moiety was in agreement with its absorption at 1633 cm^{-1} in IR, and the signal at δ_C 169.2. The DEPT results indicated two exchangeable protons, which were assigned to phenolic hydroxyls. The $FeCl_3$ positive reaction and the IR absorption at 3435cm^{-1} confirmed phenolic hydroxyl groups consistent with the singlet at $\delta_{H\text{-}3}$ 6.26 (δ_C 99.9), indicating an aromatic hydrogen *ortho-ortho* dihydroxylated. The correlation in the COSY spectrum between H-11 δ_H 1.26 (t, *J* 7.0 Hz) and H-10 δ_H 4.22 (q, *J* 7.0 Hz) evidenced an ethyl radical, and the heteronuclear correlation $^3J_{CH}$ between δ_H 4.22 (H-10) and δ_C 169.2 (C-7) confirmed the presence of an ethyl ester.

The complete attribution of the hydrogen and carbon peaks was accomplished based on the data of 2D NMR (*g*HMQC, *g*HMBC, NOESY, *g*COSY). In the *g*HMBC spectrum of **1**, correlation of H-3 with C-1 and C-5, and H-9 with C-4 and C-6 allowed to assign the other substituents in the aromatic ring. In the NOESY spectrum of **1**, a correlation between δ_H 2.10 (H-8) and δ_H 1.95 (H-9), confirmed the proximity of these methyls. Based on these data, the structure of **1** was established. This compound was previously published as a synthetic intermediate[3-5] and, to our knowledge, is being reported at first time as a natural product along with its 1D and 2D NMR data.

Compound **2** was isolated as an yellow solid and it was assigned the molecular formula $C_{13}H_{12}O_5$ (eight insaturation degrees) based on HRESIMS $[M + H]^+$ at *m/z* 249.0923], ^{13}C NMR and DEPT data. The ^1H NMR spectrum (Table 1) of **2** showed two methyl resonances at δ_H 1.42 (d, *J* 7.0 Hz, 3H) and 1.97 (s, 3H), two vinilic protons at δ_H 4.68 (s, 1H) and δ_H 4.74 (s, 1H), an aldehyde at δ_H 10.11(s, 1H) and one benzylic methine at 4.51 (q, *J* 7.0 Hz, 1H).



1



2

The ^{13}C NMR and DEPT spectra (Table 1) displayed eight sp^2 carbons (six attributed to aromatic carbons, two of them bonded to hydroxyls, and two olefinic) and two carbonyls, one assigned to lactone and another to aldehyde.

The substituents in the aromatic ring were positioned by the heteronuclear correlations $^2J_{CH}$ and $^3J_{CH}$ in *g*HMBC, between H-13/C-4/C-3/C-5 and H-11/C-6/C-5. The upfield observed at C-13 (6.3) is an indicative that this methyl is surrounded by two oxygenated carbons C-3 (166.8) and C-5 (168.5). The hydrogen-bonded phenolic OH was placed *ortho* to the lactone substituent, based on its ^1H NMR chemical shift. The connectivities between the subunits $CH_3CHC{=}CH_2$ and the aromatic ring were deduced from *g*HMBC correlations of H-12/C-7/C-8/C-9. The spatial correlations observed in 1D NOESY of H-11 with H-8/H-12 and H-12 with H-11/H-10 aided in the structural confirmation of phomopsilactone (**2**).

The known substance 2-hydroxyphenylacetic acid (**3**) was identified by comparison of the physical constants and spectroscopic data with those published in literature.[6]

Phomopsilactone (**2**) is a new substance that surprisingly contains a δ-methylene lactone subunit analogous to isotoralactone, a metabolite isolated from the plant *Cassia obtusifolia*.[7] This polyketide presents sclerotinin skeleton, whose biosynthesis was proposed by Barber *et al.*[8] and Tokoroyama and Kubota.[10]

The antifungal activity of compounds **1-3** against the phytopatogenic fungi *Cladosporium cladosporioides* and

Table 1. ^1H and ^{13}C NMR (500 and 125 MHz, δ value, *J* in Hz) spectral data and *g*HMBC correlations of **1**[a] and **2**[b]

	1			**2**		
Position	^1H	^{13}C	*g*HMBC (H to C)	^1H	^{13}C	*g*HMBC (H to C)
1		112.2			167.8	
2		154.5			100.6	
3	6.26 (s)	99.9	1, 5		166.8	
4		157.2			112.4	
5		113.8			168.5	
6		135.9			110.5	
7		169.2			150.9	
8	2.10 (s)	17.1	1, 5, 6	4.51 (q; 7.0)	33.4	7, 12, 2
9	1.95 (s)	11.1	4, 5, 6		157.4	
10	4.22 (q; 7.0)	60.2	7, 11	4.68 (s) / 4.74 (s)	97.4	9, 8 / 9, 8
11	1.26 (t; 7.0)	14.1	10	10.11 (s)	194.6	5, 6
12				1.42 (d; 7.0)	25.2	7, 8, 9
13				1.97 (s)	6.3	3, 4, 5

[a] in CD_3OD, [b] in DMSO-d_6.

C. sphaerospermum was evaluated by bioautography[11,12] and the detection limit for the compounds **1** and **2** was 1.0 μg, the same as for the positive control nystatin.

Citotoxity of the compounds **1**, **2** and **3** against human cervical tumor cell line (HeLa) were tested using the MTT assay.[13] Compound **2** exhibited weak cytotoxity (IC_{50} 200 μmol L^{-1}) and **3** a strong cytotoxicity (IC_{50} 10 μmol L^{-1}). Cisplatin, a cytotoxic agent, was used as positive control with IC_{50} 5 μmol L^{-1}.

Experimental

Instrumental and chromatography materials

Optical rotations were measured in MeOH using a Perkin Elmer polarimeter with a sodium lamp at 598 nm and 25ºC. IR spectra were recorded on a Perkin Elmer-FT-IR, using KBr pellets. The NMR spectra were recorded in CD_3OD and DMSO-d_6, on a Varian Unit 500 spectrometer at 500 and 125 MHz. Mass spectra ESI-MS were obtained on a Fisons Platform VG mass spectrometer at 20 eV. For HRESIMS a Q-TOF Autospec-Micromass equipment was used. Column chromatography (CC) was performed over reversed-phase silica gel 230-400 mesh (Merck). TLC was performed using silica gel 60 (>230 mesh) and precoated silica gel 60 PF_{254} (Merck). Spots on TLC were visualized under UV light and by spraying with anisaldehyde-H_2SO_4 reagent, followed by heating at 120 ºC. Analytical HPLC was performed on a Varian Pro Star 230 using a Phenomenex C-18 column (250 mm x 4.6 mm). Preparative HPLC was performed on a Varian Prep-Star 400 system using a Phenomenex C-18 (250 mm x 21.20 mm) preparative column.

Plant material

The leaves of *Cassia spectabilis* were collected in June 2001 in Araraquara City. The botanical identification was made by Professor Maria Cláudia Marx Young and a voucher specimen (SILVA-193) has been deposited in the Herbarium of the Instituto de Botânica de São Paulo, Brazil.

Fungal isolation

Phomopsis cassiae was isolated from healthy leaves of *Cassia spectabilis* as previously reported.[14,15] The fungus was identified by Dr. Ludwig H. Pfenning and deposited in the fungal herbarium of the Universidade Federal de Lavras, assigned as CML 292. The strain *Phomopsis cassiae* was subculture in Petri plates containing Potato Dextrose Agar (PDA) and incubated during seven days. After this period, it was inoculated at 25-28 ºC in 28 Erlenmeyer flasks of 500 mL, each containing 200 mL of Potato Dextrose Broth (PDB). The cultures were incubated at 25 ºC and aerated by agitation on an orbital shaker at 150 rpm for 28 days. Extraction of the filtered fermentation broth (*ca*. 5.6 L) with ethyl acetate (3 x 2.4 L) provided the organic phase, that was dried with $MgSO_4$ and concentrated to yield 277.1 mg of crude extract.

Extraction and isolation

The crude extract (277.1 mg) was chromatographed by CC using reverse phase silica (Merck LiChroprep® RP-18 25-40 mm; 3 x 15 cm) and eluted with H_2O:MeOH (85:15) to MeOH (100%) gradient to afford 12 fractions (150 mL each, Fr-1 to Fr-12). The fraction Fr-2 (19.3 mg, H_2O:MeOH - 65:35) was purified on a preparative HPLC column (RP - 18 H_2O:ACN - 86:14, 10 mL min^{-1}), yielding **3** (3.0 mg, t_R = 35 min). The fractions Fr-9 (4.0 mg, H_2O:MeOH - 35:65) and Fr-12 (4.6 mg, H_2O:MeOH - 75:25), were analyzed by analytical HPLC [H_2O:ACN (35:5) to ACN (100%) gradient, 40 min.] yielding **1** (4.0 mg, t_R = 26.3 min) and **2** (4.6 mg, t_R = 31.8 min).

Antifungal assay

The microorganisms used in the antifungal assays *C. cladosporioides* (Fresen) de Vries SPG 140 and *C. sphaerospermum* (Penzig) SPC 491, have been maintained at the Instituto de Botânica, São Paulo, Brazil and assays were performed using direct autobiography.[11,12] Nystatin was used as positive control (detection limit 1μg).

Cytotoxicity bioassay

The human cervical cancer cell line (HeLa) assay was performed as previously described.[13] Cisplatin was used as positive control (IC_{50} 5.0 μmol L^{-1}).

*Ethyl 2,4-dihydroxy-5,6-dimethylbenzoate (**1**).* White solid, (R_f 0.75 on SiO_2-TLC [$CHCl_3$:MeOH (9:1)]; ESI-MS, +20 eV, *m/z* (%): 211 $[M + H]^+$ (100); IR (KBr) ν_{max}/cm^{-1}: 3435, 2928, 2864, 1633; ^1H and ^{13}C NMR spectra (Table 1).

*Phomopsilactone (**2**).* Yellow solid, R_f 0.58 on SiO_2-TLC [$CHCl_3$-MeOH (9:1)]; $[\alpha]^{25}$ +50 (c 0.19 $CHCl_3$); HRESIMS: *m/z* 249.0923 (calc. for $C_{13}H_{12}O_5$, 249.0763); IR (KBr) ν_{max}/cm^{-1}: 3435, 2925, 2860, 1633; ^1H and ^{13}C NMR spectra (Table 1).

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