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Benzopyrans from *Curvularia* sp., an endophytic fungus associated with *Ocotea corymbosa* (Lauraceae)

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Abstract

An isolate of *Curvularia* sp. was obtained from the leaves of *Ocotea corymbosa*, a native plant of the Brazilian Cerrado. The ethyl acetate extract from culture of this fungus afforded two benzopyran derivatives: (2′*S*)-2-(propan-2′-ol)-5-hydroxy-benzopyran-4-one (**2**) and 2,3-dihydro-2-methyl-benzopyran-4,5-diol (**4**); and two known benzopyrans: 2-methyl-5-methoxy-benzopyran-4-one (**1**) and (2*R*)-2,3-dihydro-2-methyl-5-methoxy-benzopyran-4-one (**3**). The structures of **2** and **4** were established on the basis of comprehensive spectroscopic analysis, mainly using 1D and 2D NMR experiments. The benzopyrans **1** and **2** showed weak in vitro antifungal activity against *Cladosporium sphaerospermum* and *C. cladosporioides*. Analyses of the biological activities were also carried out on HeLa (human cervix tumor) and CHO (Chinese hamster ovary) cells, aiming to evaluate their potential effects on mammalian cell line proliferation. Results from both cell lines indicated that compound **2** was able to induce cell proliferation: 70% on HeLa cells and 25% on CHO cells.

Keywords: *Curvularia*; Endophytic fungi; Metabolites; Benzopyrans; Cell proliferation

1. Introduction

The genus *Ocotea* is well known as a source of shikimate-derived aromatics such as benzylisoquinoline alkaloids, neolignans and pyrones (Franca et al., 1975; Barbosa-Filho et al., 1989; Mandarino, 1985). Members of this genus are also widely used by Brazilian natives due to its purported pharmacological properties and resistance of the wood to fungal attacks (Chavez et al., 1995). As part of our studies on bioactive agents from metabolites produced by endophytic fungi associated with species of native plants from the Brazilian Cerrado, we initially selected 16 species of plants, which had already been phytochemically profiled by our group.

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Those species were submitted for isolation of endophytic fungi and 82 pure isolates were obtained, preserved, cultivated in liquid media and extracted with ethyl acetate to obtain crude extracts.

The extract of a *Curvularia* sp. isolated from *Ocotea corymbosa* (Meissn.) Mez. showed moderate antifungal activity against *Cladosporium sphaerospermum* and *C. cladosporioides*, triggering further studies. The crude EtOAc extract afforded two new benzopyran derivatives (**2** and **4**) and the known compounds **1** and **3**, which were tested in cell proliferation and antifungal assays. Compound **2** caused a potent proliferative stimulus to two mammalian cell lines and a weak antifungal activity. The dualistic activities described in this study (stimulatory mammalian cell proliferation and antifungal) might be of special interest in what concerns to species specificity action.

2. Results and discussion

The known compounds 2-methyl-5-methoxy-benzopyran-4-one (**1**) and (2*R*)-2,3-dihydro-2-methyl-5-methoxy-benzopyran-4-one (**3**), were identified by comparing their physical and spectroscopic data with literature values (Nagasawa et al., 1988; Wu et al., 1999). A positive optical rotation displayed by compound **3**, further allowed the assignment of the absolute configuration at C-2 as *R*.

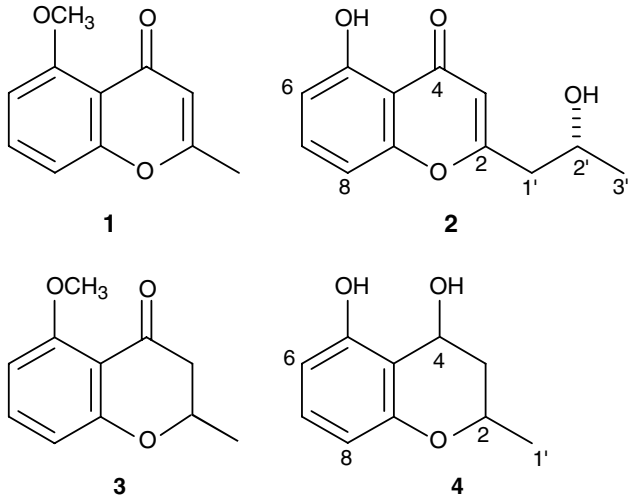
Compound **2** was isolated as a white amorphous powder and its HRESI mass data suggested the molecular formula $C_{12}H_{13}O_4$ ($[M + H]^+$ at *m/z* 221.0917). Analysis of the NMR, IR and UV spectra, suggested the presence of a chromane derivative (Saengchantara and Wallace, 1990). The ^{1}H NMR spectrum of **2** (Table 1) showed a signal at δ 12.70 (1H, *s*) attributed to a chelated phenolic OH. Spin-systems corresponding to a 1,2,3-trisubstituted benzene ring and a 2-hydroxypropyl group were also observed, along with an isolated olefin proton signal. The ^{13}C NMR spectrum showed 12 signals (Table 1) consistent with these units and with the presence of a carbonyl group. The ^{13}C NMR chemical shifts indicated that the benzene ring is 1,3-dioxygenated and that the carbonyl group is part of a benzopyran unit. Data from the *g*HMBC experiment (Table 1) supported these conclusions and enabled completion of structure **2**. For example, correlations of the H-1a′ proton with olefinic carbons C-2 and C-3 enabled location at the 2-hydroxypropyl group at C-2. In addition, the selected irradiation 1D-NOESY experiment was conducted on H-3 and showed NOE correlations with H-1a′ and H-2′. The absolute configuration of analogue (2′*S*)-7-hydroxy-2-(2′-hydropropyl)-5-methyl-chromone has been determined as *S* (Kashiwada et al., 1984) and showed a positive optical rotation. Since **2** also exhibited positive specific rotation, C-2′ was assigned the *sinister* configuration. Based on these and other data and δ values, the structure of **2** was defined as (2′*S*)-2-(propan-2′-ol)-5-hydroxy-benzopyran-4-one.

Compound **4** was obtained as a brown amorphous powder. Its HRESI mass spectrum exhibited a $[M - H_2O + H]^+$ at *m/z* 163.0757 corresponding to a molecular formula $C_{10}H_{11}O_2$. The absence of a carbonyl band in the IR spectrum suggested reduction at this position relative to **3**. The ^{1}H and ^{13}C NMR spectroscopic data (Table 1) revealed the presence of the same 1,3-dioxygenated; 1,2,3-trisubstituted aromatic system for **2** along with a spin-system corresponding to the C-2, C-3, C-4 and C-1′ portion of structure **4**. The equatorial and axial orientations of H-4, H-2, H-3 and H-1′ were

Table 1
^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectroscopic data for compounds **2** and **4** in DMSO-d_6[a]

Position	**2**				**4**		
	^{1}H (δ)	^{13}C (δ)	*g*HMBC	NOESY	^{1}H (δ)	^{13}C (δ)	*g*HMBC
2	–	170.0	–	–	^{ax}4.24 *m*	66.6	C-9
3	6.32 *s*	109.5	C-1′, C-10	H-1′a, H-2′	^{ax}1.50 *ddd* (14.7, 14.7, 4.0)	38.0	–
			–	–	^{eq}1.88 *ddd* (14.7, 4.0, 4.0)		–
4	–	182.0	–	–	^{eq}4.72 *br s*	57.1	C-10
5	–	159.9	–	–	–	156.8	–
6	6.79 *d* (8.0)	110.7	C-10	–	6.20 *dd* (8.0, 1.0)	106.9	–
7	7.63 *t* (8.0)	135.6	C-5, C-9	–	6.92 *t* (8.0)	128.7	–
8	7.03 *d* (8.0)	107.3	C-10	–	6.33 *dd* (8.0, 1.0)	106.5	–
9	–	156.3	–	–	–	156.0	–
10	–	109.9	–	–	–	112.4	–
1′*a*	2.68 *d* (8.5)	43.5	C-2, C-3	H-3	^{eq}1.32 *d* (6.0)	21.2	C-2, C-3
1′*b*	2.76 *d* (4.5)		–	–	–	–	–
2′	4.10 *m*	64.2	–	H-3	–	–	–
3′	1.18 *d* (6.0)	23.4	C-1′	–	–	–	–
5-OH	12.70 *s*	–	–	–	–	–	–
2′-OH	4.92 *d* (4.5)	–	–	–	–	–	–

[a] Chemical shifts (relative to TMS) are in (δ) ppm, coupling constants in Hz in parentheses. Assignments were aided by ^{1}H–^{1}H *g*COSY and *g*HMQC.



Scheme 1.

deduced from their coupling constants. Small *J*-values showed by H-4 suggested *vicinal eq–eq* and *eq–ax* couplings with H_2-3. The H-3_{eq} showed constants that suggested a *vicinal* coupling *eq–eq* (4.0 Hz) with H-2 and *eq–ax* (4.0 Hz) with H-4 and a *geminal* coupling (14.7 Hz) with H-3_{ax}. The 14.7, 14.7 and 4.0 Hz *J*-values for H-3_{ax} indicated a *geminal* coupling, a *vicinal ax–ax* coupling with H-2 and an *ax–eq* coupling with H-4, respectively. Based on the conformational preference of methyl group over hydroxyl, the methyl group at C-2 was assumed to be in an equatorial position on a half-chair ring, which is the preferred conformation for benzopyrans (Joshi and Merchant, 1984). Therefore, due to equatorial position determined for H-4, compound **4** was characterized as the *trans*-isomer at H-4-H-2. All NMR signals were assigned by analysis of *g*HMQC and *g*HMBC experiments (Table 1) and δ values. Compound **4** was thus identified as the previously unreported 2,3-dihydro-2-methyl-benzopyran-4,5-diol.

Compounds **1**–**4** (see Scheme 1) were evaluated against *Cladosporium sphaerospermum* and *C. cladosporioides* using direct bioautography (Rahalison et al., 1991). Compounds **1** and **2** exhibited a weak antifungal activity, showing a detection limit of 10 μg for both substances. Compound **3** and **4** did not show any antifungal activity. Nystatin was employed as a positive control showing a detection limit of 1 μg. The reduction at $\Delta^{2,3}$ in **3**, changing the conformation of the pyrane ring, may be the cause of the loss of the activity.

Biological evaluation of **1**–**4** was also carried out using HeLa (human cervix tumor) and CHO (Chinese hamster ovary) cell lines. Compound **2**, at 20 μmol/L was able to induce 70% increase in cell proliferation to HeLa and 25% to CHO cells, when compared to the DMSO treated cells. The compounds **1**, **3** and **4** were inactive in CHO and displayed a weak stimulatory effect in HeLa cells. When cisplatin, a well known antineoplastic agent, was used as a control, a clear inhibition of cell proliferation was observed.

The biological activity of **1** and **2** seems to be differentially specific to fungi or mammalian cells, and also to different mammalian cell lines. The indication of a possible specific mechanism of action is of great interest and deserves further investigation.

3. Experimental

3.1. General experimental procedure

Optical rotations were measured in MeOH using a Perkin–Elmer polarimeter with a sodium lamp operating at 598 nm and 25 °C. UV spectra were recorded on a Shimadzu UV-2401 PC spectrophotometer. IR spectra were run on a Nicolet Impact-400 spectrophotometer. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectra were recorded on a VARIAN DRX-500 spectrometer, using TMS as an internal standard. HRESI mass spectra were obtained on an Autospec-Micromass mass spectrometer. Analytical HPLC was performed on a Varian Pro Star 230 using a Phenomenex C-18 column (250 mm × 4.6 mm). Column chromatography (CC) was performed over reversed-phase silica gel 230–400 mesh (Merck). TLC was performed using Merck silica gel 60 (>230 mesh) and precoated silica gel 60 PF_{254}. Spots on TLC plates were visualized under UV light and by spraying with anisaldehyde-H_2SO_4 reagent followed by heating at 120 °C. Preparative HPLC was performed on a Varian Prep-Star 400 system using a Phenomenex C-18 (250 mm × 21.2 mm) preparative column.

3.2. Plant material

Authenticated *O. corymbosa* plant material was collected in ''Estação Experimental de Araraquara'', Araraquara, São Paulo, Brazil, in 2001. The botanical identification was made by Professor Maria Cláudia Marx Young and a voucher specimen was deposited at the Herbarium of the Botanic Garden of São Paulo, Brazil (Voucher No MCMY-1314).

3.3. Isolation of the endophytic fungus

For the isolation of the endophytic fungus, adult and healthy leaves were selected and submitted to surface sterilization. They were first washed with water and soap, and immersed in a 1% aqueous sodium hypochlorite solution for 5 min and 70% aqueous ethanol for 1 min. A second washing with water and soap was performed and finally the leaves were immersed in sterile water for 10 min. The sterilized leaves were cut into 2 × 2 cm pieces and deposited on a Petri dish containing PDA (potato–dextrose–agar) and anthamicine sulfate

(50 mg/mL) with approximately three to four pieces on each dish. The material was incubated at 25 °C for 10 days and the endophyte *Curvularia* sp. was isolated by replication and preserved in sterile water (Maier et al., 1997). The fungus was identified by Dr. José Odair Pereira (UFA) and deposited in NuBBE's collection with culture number "PAJ-04".

3.4. Growth of Curvularia and production of the EtOAc extract

The fungus was cultivated on a small scale in 2 Erlenmeyer flasks (500 mL), each containing 0.8 g of potato extract, 4.0 g dextrose and 200 mL distilled water (PDB) which were autoclaved at 125 °C for 15 min. Approximately 10 small pieces (1 × 1 cm) of PDA medium, from the Petri dish containing biomass of the *Curvularia* isolated were inoculated into each flask and the flasks were sealed with cotton to permit aerobic growth. After incubation for 28 days at 25 °C on rotary shakers at 150 rpm, the mycelia biomass accumulated in the flasks was separated from the aqueous medium by filtration, and the filtrate was partitioned with ethyl acetate (3 × 200 mL). Collection and evaporation of the organic phase in vacuo yielded a brown solid residue (158 mg). After this extract was found to display bioactivity, this process was scaled up using 4.0 L of PDB (20 Erlenmeyer flasks) and afforded 1.8 g of EtOAc extract.

3.5. Antifungal assay

The microorganisms *C. cladosporioides* (Fresen) de Vries SPC 140 and *C. sphaerospermum* (Perzig) SPC 491 were used in the antifungal assay. They have been maintained at the Instituto de Botânica, São Paulo, SP, Brazil. The compounds **1**–**4** were applied on a precoated Si-gel TLC plates using a solution (10 μL) containing 100, 50, 25, 10, 5 and 1 μg. After eluting with $CHCl_3$:CH_3OH (9:1) they were sprayed with the fungi (Rahalison et al., 1991). Nystatin was employed as positive control.

3.6. Determination of cell viability after treatment with benzopyrans

HeLa (human cervix tumor) and CHO (Chinese hamster ovary) cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2, using Dulbecco's modified Eagle's medium (DMEM, Life Technologies Inc., Gaithersburg, MD) supplemented with 10% Fetal Calf Serum (FCS, Life Technologies Inc., Gaithersburg, MD). Penicillin (100 μg/mL) and streptomycin (100 μg/mL) were included in medium to prevent bacterial growth. A stock solution (20 mmol/L) was prepared by dissolving compounds **1**–**4** in DMSO (vehicle). The final concentrations (200, 20 and 2 μmol/L) were achieved by direct dilution into the cell medium. Cells were plated (10^4 cells/well) in a 48-well tray 24 h prior to the beginning of the experiment. After addition of the compound or the vehicle, cells were analyzed after a period of 48 h using the MTT assay (Mosmann, 1983; Rubinstein et al., 1990).

3.7. Extraction and isolation

The EtOAc extract (1.8 g) was fractionated by column chromatography (CC) using reversed-phase silica gel (ODS) and eluted with a MeOH–H_2O gradient (1:9 100% MeOH) affording 8 fractions (A–H). Fraction A (125 mg) was further purified using reverse phase prep. HPLC [λ = 221 nm, 12.0 mL/ min., CH_3CN:H_2O (2:8)] affording **1** (2.2 mg) and **2** (1.8 mg). Fraction B (100 mg) was also purified using reversed phase prep. HPLC [λ = 230 nm, 12.0 mL/min., CH_3CN:H_2O (23:77)], affording **3** (10.2 mg) and finally fraction C (770 mg) was also submitted to reverse phase prep. HPLC, [λ = 221 nm, 12.0 mL/min., CH_3CN:H_2O (37:63)] to afford compound **4** (2.7 mg).

3.7.1. (2′S)-2-(propan-2′-ol)-5-hydroxy-benzopyran-4-one (*2*)

Amorphous powder (1.8 mg); $[\alpha]_D^{25}$ +30 (*c* 0.25, MeOH); UV λ_{max} (CH_3OH) nm (log ε): 240 (4.0); IR ν_{max} (KBr) cm^{-1} : 3732, 1629, 1030. For ^1H and ^{13}C NMR spectroscopic data, see Table 1; HRESIMS: *m/z* 221.0917 $[M + H]^+$ Calcd. for $C_{12}H_{13}O_4$, 221.0814.

3.7.2. 2,3-dihydro-2-methyl-benzopyran-4,5-diol (*4*)

Brown amorphous powder (2.7 mg); $[\alpha]_D^{25}$-15 (*c* 0.25, MeOH); UV λ_{max} (CH_3OH) nm (log ϵ): 240 (3.8); IR ν_{max} (KBr) cm^{-1}: 3730, 1033. For ^1H and ^{13}C NMR spectroscopic data, see Table 1; HRESIMS: *m/z* 163.0757 $[M + H]^+$ Calcd. for $C_{12}H_{13}O_4$, 163.0590.

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