

## Novel antimicrobial secondary metabolites from a *Penicillium* sp. isolated from Brazilian cerrado soil

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**Keywords:** antibacterial activity, cerrado, naphthalenoids, *Penicillium*, secondary metabolites.

**Abbreviations:** CYA: czapek yeast extract agar  
DEPT: distortionless enhancement by polarization transfer  
DMF: dimethylformamide  
EIMS: electron-ionization mass spectrometry  
EtOAc: ethylacetate  
G25N: 25% glycerol nitrate agar  
HMBC: heteronuclear multiple bond correlation  
HMQC: heteronuclear multiple quantum coherence  
MEA: malt extract agar  
MIC: minimum inhibitory concentration  
NMR: nuclear magnetic resonance  
NOESY: nuclear overhauser and exchange spectroscopy  
TLC: thin layer chromatography

The morphological features of a *Penicillium*, isolated from Brazilian cerrado soil, were characterized and showed to be distinctly different from all well-defined *Penicillium* species. Chemical and biological investigation on the ethyl acetate extract of this *Penicillium* isolate resulted in the isolation of three new naphthalenoids: a major metabolite, methyl 6-acetyl-4-

methoxy-5, 7, 8-trihydroxynaphthalene-2-carboxylate and two minor ones, methyl 6-acetyl-4-methoxy-7,8-dihydroxynaphthalene-2-carboxylate and methyl 6-acetyl-4-methoxy-5, 8-dihydroxynaphthalene-2-carboxylate. Their structures were determined based on their mono and bidimensional nuclear magnetic resonance data. Acetyl, allyl and methoxyl derivatives of

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the major metabolite were prepared in order to establish structure-activity relation. Antimicrobial activity of the major natural product and its semi-synthetic derivatives was screened by macro dilution methodology and the corresponding minimum inhibitory concentrations were determined. Natural secondary metabolite methyl 6-acetyl-4-methoxy-5,7,8-trihydroxynaphthalene-2-carboxylate, isolated in a very high yield ( $0.3175 \text{ mg.L}^{-1}$ ) showed to be the most active compound, possessing expressive activity against *Candida albicans* (minimum inhibitory concentration (MIC)  $32 \text{ }\mu\text{g/mL}$ ), *Listeria monocitogenes* and *Bacillus cereus* (MIC  $64 \text{ }\mu\text{g/mL}$  for both).

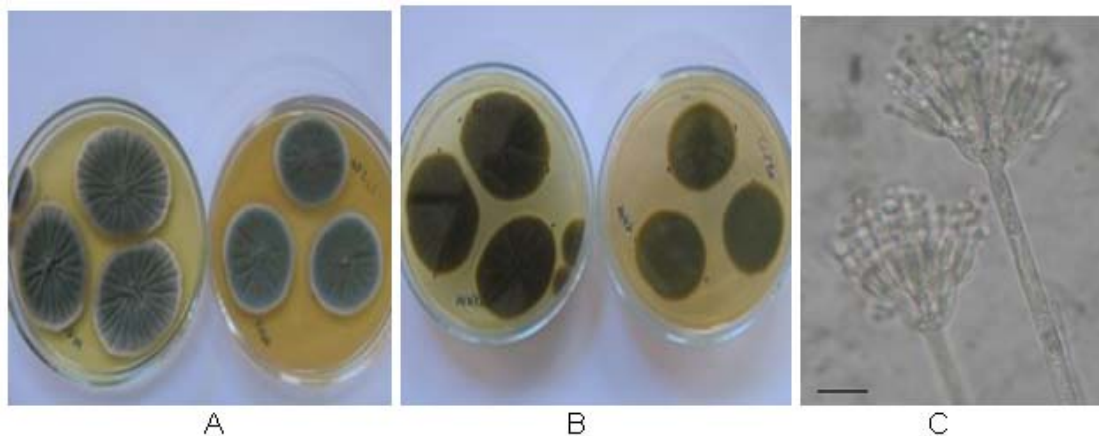
Fungi play a major role in soil ecosystems along with bacteria, protists, small invertebrates and plants, through complex trophic interactions. Most soil fungi are regarded as saprobes, decomposing organic matter and contributing to nutrient cycling, while several species form mycorrhizal associations with plants or are plant pathogens (Pfenning and Abreu, 2006). Also recognized as prolific secondary metabolite producers, fungi have provided several bioactive compounds and chemical models currently used as pharmaceuticals, and soils are traditionally the main source of fungal genetic resources for bioprospection programs (Adrio and Demain, 2003). Despite that, the biodiversity and biotechnological potential of the soil mycobiota in many tropical regions is still poorly studied.

Cerrado is a savannah-like ecosystem that occupies around 20% of the Brazilian territory. It is characterized by a seasonal climate, old and weathered acidic soils with low nutrient and high aluminum content, and sparse vegetation. This quite hostile environment is thought to select species with adapted metabolism and good potential for delivering new bioactive metabolites. Indeed, the antimicrobial activity of several fungal species isolated from the cerrado

soil towards pathogenic bacteria has been recently described by our group (Takahashi et al. 2008).

*Penicillium* is a large anamorphic (asexual state) ascomycetous fungal genus with widespread occurrence in most terrestrial environments. This genus comprises more than 200 described species and many are common soil inhabitants, as well as food borne contaminants or food ingredients used in the preparation of cheese and sausages (Pitt, 2000; Frisvad and Samson, 2004). *Penicillium* species produce a much diversified array of active secondary metabolites, including antibacterial (Rancic et al. 2006; Lucas et al. 2007), antifungal substances (Nicoletti et al. 2007), immunosuppressants, cholesterol-lowering agents (Kwon et al. 2002), and also potent mycotoxins (Frisvad and Samson, 2004). Thousands of *Penicillium* isolates have probably been screened in bioprospecting programs since the discovery of penicillin, and new bioactive metabolites continue to be discovered from these fungi nowadays (Larsen et al. 2007; Ge et al. 2008; Takahashi and Lucas, 2008), indicating their current importance as sources of high amounts of novel bioactive molecules to be used by pharmaceutical industry.

We have been screening a collection of filamentous fungi isolated from Serra do Cipó National Park soil, a preserved area of cerrado vegetation (Takahashi et al. 2008) for novel fungal natural products targeting at metabolites with biotechnological applications for the pharmaceutical industry. This fungal collection is mostly constituted by *Penicillium* species, such as *Penicillium slerotiorum*, *Penicillium simplicissimum*, *Penicillium brasilianum*, *Penicillium janthinellum* and *Penicillium waksmanii* and was fully tested for antibacterial activity (Takahashi et al. 2008). One of the most active extracts was obtained from a single *Penicillium* isolate with atypical morphological characteristics. From this isolate, three novel compounds



**Figure 1. Colony appearance and micromorphology of *Penicillium* sp.**

A - Colony appearance (obverse) after 7 days at 25°C on CYA (left) and MEA (right);  
 B - Colony appearance (reverse) after 7 days at 25°C on CYA (left) and MEA (right);  
 C - Conidiophores (original magnification 1000 X). Bar, 10  $\mu\text{m}$ .

Table 1. NMR spectroscopic data ( $\delta$  from TMS, multiplicity) for compounds 1, 2 and 3.

Position	1 (CDCl <sub>3</sub> /0.1%DMSO-d <sub>6</sub> )		2 (MeOD-d <sub>4</sub> )		3 (DMSO-d <sub>6</sub> )	
	$\delta_H$	$\delta_C$	$\delta_H$	$\delta_C$	$\delta_H$	$\delta_C$
1	7.07, d (J= 2Hz)	108.56	6.95, d (J= 2.1Hz)	109.02	6.97, d (J= 2.1Hz)	109.09
2		128.69		130.10		130.10
3	6.65, d (J= 2Hz)	103.65	6.65, d (J= 2.1Hz)	104.46	6.67, d (J= 2.1Hz)	104.46
4		157.16		159.37		158.86
5		158.86	6.12, s	108.95		149.41
6		112.94		112.80		112.80
7		142.05		149.41	6.23, s	109.78
8		155.08		160.70		160.07
9		110.67		111.00		112.00
10		125.58		127.85		127.85
CH <sub>3</sub> -6	2.64, s	18.96	2.19, s	22.18	2.29, s	21.18
OCH <sub>3</sub> -2	3.72, s	52.23	3.70, s	52.62	3.70, s	52.71
OCH <sub>3</sub> -4	3.70, s	56.13	3.66, s	56.70	3.68, s	56.70
CO-2		166.17		163.44		168.06
CO-6		200.31		202.31		202.31
OH-5, 7, 8	10.8 (2H) and 9.51 (1H), br s					

s= singlet; d= doublet; br s= broad singlet

(1-3) were isolated, identified and tested for antibacterial and antifungal activities, along with three semi-synthetic derivatives prepared from compound 1.

## MATERIALS AND METHODS

### General experimental procedures

Column chromatography was performed on silica gel (Merck, Darmstadt, Germany) (230-400 mesh). Thin layer chromatography (TLC) was performed on commercial TLC plates (pre-coated Kieselgel 60 F<sub>254</sub> TLC, 20 x 20 mm, thickness 0.25 mm, Merck). Culture medium was prepared

with glucose (Labsynth, Brazil), peptone (Isofar 2115, Brazil) and yeast extracts (Biobras, Brazil). Solvents used for chromatography (column and thin layer) were purchased from Grupo Química (São Paulo, Brazil). Deuterated solvents, reagents and dimethylformamide (DMF) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Chloramphenicol and miconazole were purchased from Sigma Chemical Co. (St. Louis, MO). Infrared spectra were recorded on a Perkin Elmer 283b spectrometer. The samples were analyzed as KBr pellets. Electron-ionization mass spectrometry (EIMS) data were recorded on a Kratos concept II H mass spectrometer. <sup>1</sup>H

nuclear magnetic resonance (NMR) and  $^{13}\text{C}$  NMR spectra were obtained on Bruker model DRX 400 avance with standard pulse sequences operating at 400 MHz for  $^1\text{H}$  NMR and 100 MHz for  $^{13}\text{C}$  NMR.

### Isolation and characterization of the fungal isolate

As part of a program aiming at the identification of antibiotic producing fungi, around 200 fungal isolates were recovered from 15 soil samples randomly collected from an area of native cerrado (Brazilian savannah) in the Serra do Cipó National Park, Minas Gerais State of Brazil. The fungi were isolated by soil dilution plate method and preserved at  $4^\circ\text{C}$  for further characterization (Takahashi et al. 2008). Among several *Penicillium* isolates recovered, one exhibited distinct cultural characteristics and was investigated in more detail.

Using the media and growth conditions specified by Pitt (2000), a wide range of morphological features of the *Penicillium* isolate were examined on Czapek yeast extract agar (CYA) at 25 and  $37^\circ\text{C}$ ; malt extract agar (MEA) at  $25^\circ\text{C}$ ; and 25% glycerol nitrate agar (G25N) at  $25^\circ\text{C}$  incubated for 7 days in darkness. Microscopic preparations made from colonies cultivated on MEA and mounted in glycerin were used for evaluation of micromorphological traits of conidiophores and conidia. Light micrographs were taken at 1000 X magnification in an Olympus CX40RF100 light microscope coupled to a Canon A620 digital camera. The cultural and morphological characteristics of the isolate were compared with species descriptions contained in the *Penicillium* manuals (Pitt, 1979; Pitt, 2000).

### Culturing of *Penicillium* sp., extraction and isolation of metabolites

Spores of *Penicillium* sp. were inoculated in a medium prepared in distilled water containing glucose (20 g/L), peptone (10 g/L) and yeast extract (5 g/L). The fungal culture (5 L) was incubated without agitation for 2 weeks at  $25^\circ\text{C}$ . Once the incubation period was complete, mycelia was filtered off, soaked on ethylacetate (EtOAc) for 24 hrs and filtered, resulting in an organic layer. The aqueous medium was submitted to a liquid-liquid extraction with EtOAc. Both EtOAc extracts were combined and concentrated under reduced pressure to yield 3.5 g of a brown solid. Mycelia and culture medium were also extracted with butanol (BuOH) following the same procedure adopted for the EtOAc extract. Both extracts were tested for antimicrobial activity using the disc diffusion assay and only the EtOAc showed encouraging bioactivity. Therefore, only this extract was further investigated.

An aliquot of 2.8 g was taken from the EtOAc extract and subjected to silica gel column chromatography. Mixtures of hexane with increasing percentages of EtOAc were used to elute the compounds. The separation led to a number of fractions from where three main fractions were further

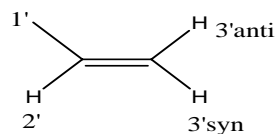
studied. The first fraction (465 mg), eluted with 100% hexane until hexane:EtOAc (8:2), was examined but proved to be mainly composed of a mixture of fatty acids and long chain alcohols. Fraction 2, eluted with hexane:EtOAc (1:1), gave compound **1** (1.27 g) after recrystallization from hexane:acetone (1:1). Fraction 3, eluted with hexane:EtOAc (4:6), led to a mixture (26 mg) of compounds **1**, **2** and **3** that was further chromatographed on preparative thin layer chromatography using hexane:EtOAc (1:1) as eluent, in order to remove compound **1**. Compound **2** and **3** were obtained as a mixture (4.8 mg), from which compound **2** was isolated by preparative thin layer chromatography in hexane:EtOAc (4:6).

**Compound 1.** [mp  $215\text{--}217^\circ\text{C}$ ; IR (KBr)  $\lambda_{\text{max}}$  3443, 3398, 1716, 1613,  $1592\text{ cm}^{-1}$ , EIMS m/z 159 ( $\text{C}_6\text{H}_7\text{O}_5$ ) (100), 129 (50), 101 (28), 143 (25), 201(15), 185 (13), 217 (10)], **2** and **3**:  $^1\text{H}$  and  $^{13}\text{C}$  NMR data (Table 1).

**Compound 4.** (Acetylation procedure): To a solution of naphthotriol **1** (0.05 mmol, 15 mg) in pyridine (1.0 mL) 1 mL of acetic anhydride was added and the reaction mixture was left at room temperature for 48 hrs. After usual work up, the triacetate was obtained as yellow needles (acetone), mp  $147\text{--}150^\circ\text{C}$ ; IR (KBr)  $\lambda_{\text{max}}$  3551, 3478, 3413, 3236, 1783, 1730, 1680, 1637 and  $1617\text{ cm}^{-1}$ .  $^1\text{H}$  NMR, ( $\text{CDCl}_3$ , 400 MHz)  $\delta$ : 7.25 (d, 1H,  $J=1.8\text{ Hz}$ ) H-1; 6.82 (d, 1H,  $J=1.8\text{ Hz}$ ) H-3; 3.71 (s, 3H)  $\text{CH}_3\text{-2}$ , 3.68 (s, 3H)  $\text{CH}_3\text{-4}$ , 2.57 (s, 3H)  $\text{CH}_3\text{-6}$ ; 2.32, 2.06 and 2.04 (s, 3H each)  $\text{CH}_3\text{-5}$ , 7, 8.  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz)  $\delta$ : 115.2 (C-1); 131.4 (C-2); 109.3 (C-3); 158.1 (C-4); 152.4 (C-5); 127.5 (C-6); 140.0 (C-7); 145.3 (C-8); 125.2 (C-9); 129.2 (C-10); 52.8 ( $\text{CH}_3\text{-2}$ ); 56.4 ( $\text{CH}_3\text{-4}$ ); 29.7 ( $\text{CH}_3\text{-6}$ ); 21.0, 19.0, 20.1 ( $\text{CH}_3\text{-5}$ , 7, 8, respectively); 165.6, 167.6, 168.8, 167.5 (CO-2, 5, 7, 8, respectively); 188.1 (CO-6).

**Compound 5.** (Allylation procedure): A solution of naphthotriol **1** (0.05 mmol, 15 mg), anhydrous  $\text{NaHCO}_3$  (15 mmol, 1.2 g) and allyl bromide (15 mmol, 1.5 mL) in acetone (40 mL) was refluxed for 24 hrs. Solvent was removed under vacuum and the residue was extracted from ethylacetate to yield the desired allyl ether as a gum.

$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz)  $\delta$  6.69 (d, 1H,  $J=2.3\text{ Hz}$ ) H-1; 6.49 (d, 1H,  $J=2.3\text{ Hz}$ ) H-3; 6.02 (m, 1H) H-7 (2'); 5.90 (m, 2H) H-5, 8 (2'); 5.41 (dd, 1H,  $J=17.21\text{ Hz}$  and  $1.3\text{ Hz}$ ) H-7 (3' anti); 5.32 (dd, 1H,  $J=10.4\text{ Hz}$  and  $1.3\text{ Hz}$ ) H-7 (3' syn); 5.22 (dd, 2H,  $J=17.21\text{ Hz}$  and  $1.4\text{ Hz}$ ) H-5, 8 (3' anti); 5.11 (dd, 2H,  $J=10.4\text{ Hz}$  and  $1.2\text{ Hz}$ ) H-5, 8 (3' syn); 4.6 (d, 2H,  $J=5.6\text{ Hz}$ ) H-7(1'); 4.4 (d, 4H,  $J=6\text{ Hz}$ ) H-5, 8 (1'), 3.81 (s, 3H)  $\text{CH}_3\text{-2}$ , 3.35 (s, 3H)  $\text{CH}_3\text{-4}$ , 2.53 (s, 3H)  $\text{CH}_3\text{-6}$ .



$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz)  $\delta$  106.6 (C-1); 131.1 (C-2); 101.4 (C-3); 160.7 (C-4); 162.3 (C-5); 133.3 (C-5,8 (2'));

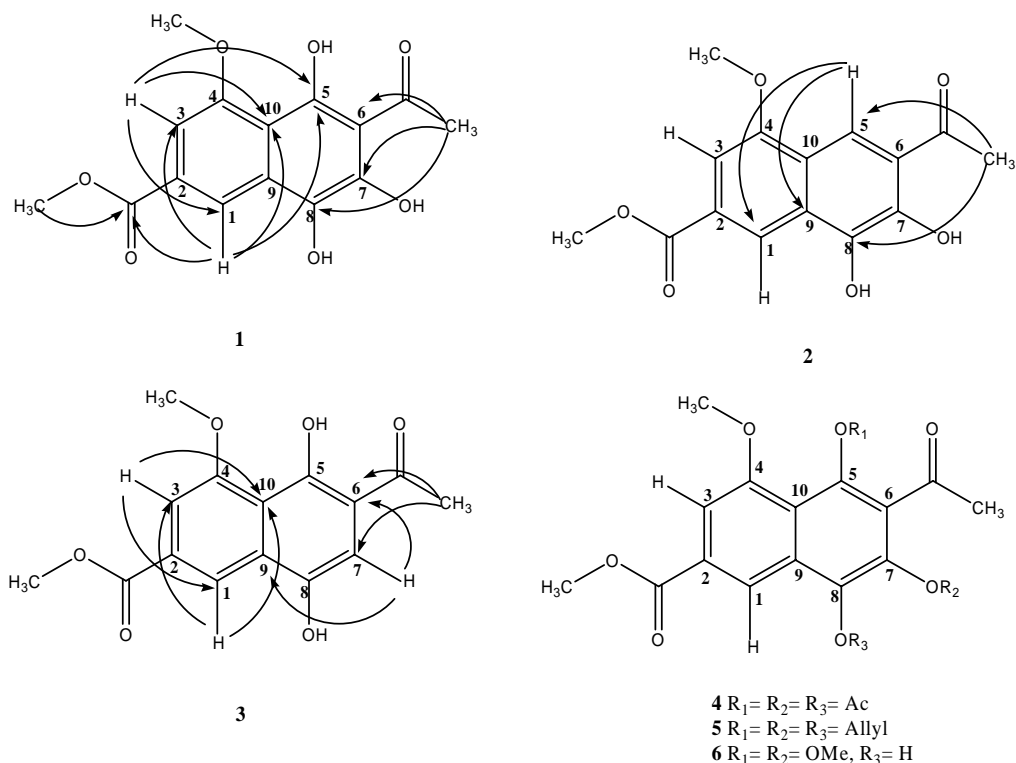


Figure 2. Structure of compounds 1-6 and selected HMBC correlations indicated by arrows.

132.2 (C-7 (2')); 118.4 (C-7 (3')); 117.8 (C-5,8 (3')); 125.0 (C-6); 137.5 (C-7); 151.4 (C-8); 125.1 (C-9); 121.3 (C-10); 75.5 (C-5,8 (1')); 69.2 (C-7 (1')); 52.7 (CH<sub>3</sub>-2); 56.2 (CH<sub>3</sub>-4); 18.5 (CH<sub>3</sub>-6); 169.3 (CO-2); 188.6 (CO-6).

**Compound 6.** (Methylation procedure): Sodium hydrogenocarbonate (5 mmol) and methyl iodide (5 mmol) were added sequentially to a solution of naphthotriol **1** (0.5 mmol) in acetone. The resulting solution was stirred at room temperature for 48 hrs to yield the desired ether after usual work up. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 100 MHz)  $\delta$ : 7.11 (d, 1H,  $J=2.3$  Hz, H-1); 6.67 (d, 1H,  $J=2.3$  Hz, H-3); 3.89 (s, 3H, CH<sub>3</sub>-2), 3.76, 3.72, 3.25 (s, 3H each, CH<sub>3</sub>-4, 5 and 7); 2.54 (s, 3H, CH<sub>3</sub>-6). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz)  $\delta$ : 105.4 (C-1); 103.0 (C-3); 129.2 (C-3); 160.5 (C-4); 157.0 (C-5); 119.1 (C-6); 143.5 (C-7); 155.0 (C-8); 115.0 (C-9); 126.8 (C-10); 52.5 (CH<sub>3</sub>-2); 60.6, 56.2, 55.8 (CH<sub>3</sub>-4, 5 and 8); 18.9 (CH<sub>3</sub>-6); 166.0 (CO-2); 199.9 (CO-6).

### Antimicrobial bioassays

**Test strains.** The test strains, *Listeria monocitogenes* ATCC 19115, *Streptococcus pyogenes* ATCC 19615, *Salmonella typhimurium* ATCC 13311, *Candida albicans* ATCC 18804 and *Bacillus cereus* ATCC 11778 used for this study were obtained from American Type Culture Collection. The strain of *Listeria* sp. was provided by Instituto de Ciências Biológicas, UFMG, Brazil.

**Bioassays.** Disc diffusion test was carried out according to standard procedure (Lana et al. 2006). Minimum inhibitory concentration (MIC) was evaluated by the broth dilution test using standard inoculums of 10<sup>5</sup> CFU mL<sup>-1</sup> (Rex et al. 2001). Serial dilutions of test compounds, previously dissolved in DMF, were prepared to final concentrations of 512, 256, 128, 64, 32, 16, 8, 4, 2, and 1  $\mu$ g in 1 mL of brain hearth infusion (BHI) medium. To each tube, 100  $\mu$ L of the inoculum was added. MIC, defined as the lowest concentration of the test compound which inhibits the visible growth after 18 hrs, was determined visually after incubation for 18 hrs at 37°C, and the results are presented in Table 2. Tests using DMF as negative control and chloramphenicol (bacteria) or miconazole (fungus) as positive control were carried out in parallel. All tests were performed in duplicate with full agreement between both results.

### RESULTS AND DISCUSSION

#### Macro- and micromorphology features of the *Penicillium* sp.

The conidiophores are biverticillate having smooth to finely rough stipes with 300 to 800  $\mu$ m in length, with inflated apices; conidiophores from the aerial mycelium are shorter. Metulae are formed in verticills of 4-6 on the top of stipes, smooth to finely rough, 9-12 (-15)  $\mu$ m in length, with

inflated apices (up to 5  $\mu\text{m}$ ). Rami are occasionally formed. Phialides are flask-shaped with short necks, 7-10 x 2-3  $\mu\text{m}$ , in number of 5 or more per metula. Conidia are green, globose to subglobose, smooth to finely rough, 2-3  $\mu\text{m}$ , arranged in columns. Structures resembling sclerotia are produced in mycelium. These structures have irregular shape, 50-300  $\mu\text{m}$  in size and are formed by a crystalline yellowish green matter mixed with hyphae.

Colonies on CYA (7 days, 25°C) were 40-45 mm in diameter, sulcate, velutinous, mycelium was white to pale at the margins and greyish in the center, sporulation was heavy with greyish green colors, reverse was dark green with cream to yellow margins, clear exudates were produced in variable amounts and a dark yellowish green diffusible pigment was produced. Colonies on MEA (7 days, 25°C) were 33-37 mm in diameter, plane to sulcate, velutinous, mycelium was white, with heavy sporulation, greyish green in the center and blue-green at the margins, reverse was dark green with cream to yellow margins. Exudates were absent; dark yellowish green diffusible pigment was produced in small amounts. Colonies on G25N (7 days, 25°C) were 8-11 mm in diameter, sulcate, elevated, velutinous, mycelium was white with sparse light green sporulation, reverse was pale, exudates and diffusible pigments were absent (the latter being produced after two weeks). No growth occurred in CYA at 37°C.

The regular production of biverticillate penicilli with 4 to 6 terminal metulae, and phialides, which were flask shaped and distinctly shorter than metulae by our *Penicillium* isolate are indicatives that it belongs to the subgenus *Furcatum* section *Furcatum* (Pitt, 2000). The growth rate on G25N in 7 days (8-11 mm) also gave support for this classification. The isolate showed cultural and micromorphological features which are very similar to those of *Penicillium raistrickii* (Pitt, 2000) (Figure 1). However, other characteristics were quite distinct from those of *P. raistrickii*, such as the production of conidiophores with smooth stipes (Figure 1), colonies with velutinous texture, the production of a dark yellowish green diffusible pigment in all culture media and the absence of true sclerotia in the mycelium. The isolate also showed some affinities with other species in the subgenus *Furcatum* such as *Penicillium paxillii* (e.g. long, smooth stipes and metulae with enlarged apices) and *Penicillium herquei* (e.g. colony reverses of green colors and production of dark yellowish green diffusible pigment in culture media) (Kornerup and Wansher, 1978; Pitt, 1979) but these affinities were not sufficient for an accurate identification. Therefore, we maintained this fungus identification only at the genus level to avoid misidentifications commonly reported for *Penicillium* species.

### Secondary metabolites isolated

An aliquot of the bioactive EtOAc extract was loaded on a silica gel chromatography column and eluted using hexane with increasing amounts of EtOAc to afford three new

naphthalenoids (**1-3**). Identification of the isolated compounds was carried out by using infrared and modern nuclear magnetic resonance techniques, distortionless enhancement by polarization transfer (DEPT), heteronuclear multiple quantum coherence (HMQC), nuclear overhauser and exchange spectroscopy (NOESY) and heteronuclear multiple bond correlation (HMBC). Compounds were characterized as being methyl 6-acetyl-4-methoxy-5,7,8-trihydroxynaphthalene-2-carboxylate (**1**), methyl 6-acetyl-4-methoxy-7,8-dihydroxynaphthalene-2-carboxylate (**2**) and methyl 6-acetyl-4-methoxy-5,8-dihydroxynaphthalene-2-carboxylate (**3**).

Compound **1**, isolated as yellow needles, exhibited, on its infrared spectrum, bands characteristic of hydroxyl (3443 and 1592  $\text{cm}^{-1}$ ), ketone (1716  $\text{cm}^{-1}$ ) and aromatic (3398 and 1613  $\text{cm}^{-1}$ ) moieties. The analysis of the  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of **1** and of DEPT experiment revealed the presence of a methyl group belonging to an acetyl function, two methoxyl groups, two carbonyls, one belonging to an acetate group and the other to an acetyl one, and three hydroxyl groups. The presence of three hydroxyl groups was confirmed by the fact that the acetylation of **1** gave triacetate **4**. The placement and the assignments of all the protons and the carbons were supported by the HMBC spectrum. Noticeable were the  $^3J$  correlations of H-3 and H-1 to C-10 and C-5, which allowed us to place C-10 at the ring junction and one hydroxyl group at the C-5 position (Figure 2, Table 1). HMBC correlations of methyl group of the acetyl function group at C-6 to C-7 and C-8 were also observed and confirmed the position of the acetyl and consequently, of the two remaining hydroxyl groups. Full structural data of **1** are summarized in Table 1.

Compound **2** was isolated as an amorphous yellow solid and fully identified by the assignments of all the protons and the carbons by comparison with data obtained for compound **1**. Structure determination was supported by a combination of 1D ( $^1\text{H}$  and  $^{13}\text{C}$  NMR and DEPT) and 2D NMR experiments (NOESY, HMQC and HMBC correlations) data, summarized in Table 1.  $^1\text{H}$  NMR spectrum showed the presence of an additional aromatic hydrogen signal at  $\delta_{\text{H}}$  6.12, in comparison to **1**, which appeared as a singlet (suggesting, in **2**, presence of only two hydroxyl groups). HMQC and DEPT experiments clearly showed signals of three hydrogens bounded to aromatic carbons (C-1, C-3 and C-5). The placement of the additional hydrogen at C-5, rather than at C-7 or C-8, was supported by the NOESY which showed proximity between H-5 and the methoxyl and the acetyl groups respectively in positions 4 and 6 (Figure 2). This assignment was supported by the HMBC correlations of H-5 to C-1 and C-9 (Figure 2).

Compound **3** was obtained in a mixture (4.8 mg) along with compound **2** and its spectral analysis was performed based on the 1D and 2D NMR data obtained for the mixture. A close comparison of compounds **2** and **3** data led to the attribution of all the signals that are summarized in Table 1.

Table 2. Minimum inhibitory concentration (MIC) of the selected compounds.

Strain	MIC ( $\mu\text{g mL}^{-1}$ )					
	1	4	5	6	C <sup>a</sup>	M <sup>b</sup>
<i>B. cereus</i> <sup>a</sup>	64 (64)	128 (128)	nt	256 (256)	32 (32)	nt
<i>L. monocitogenes</i> <sup>a</sup>	64 (64)	32 (32)	512 (512)	256 (256)	32 (32)	nt
<i>S. pyogenes</i> <sup>a</sup>	128 (128)	128 (128)	512 (512)	512 (512)	32 (32)	nt
<i>Listeria sp</i> <sup>a</sup>	128 (128)	128 (128)	256 (256)	256 (256)	32 (32)	nt
<i>S. typhimurium</i> <sup>a</sup>	128 (128)	128 (128)	nt	256 (256)	32 (32)	nt
<i>C. albicans</i> <sup>b</sup>	32 (32)	64 (64)	256 (256)	256 (256)	nt	32 (32)

<sup>a</sup> C: Chloramphenicol. <sup>b</sup> M: Miconazole. nt: not tested. Values in parenthesis ( ) refer to the value of the duplicate sample reading.

The general features of the <sup>1</sup>H and <sup>13</sup>C NMR spectra of compound **3** showed a resemblance to those of compound **2**, suggesting it to be a positional isomer of **2**. This observation was consistent with the results obtained from DEPT experiment. The HMBC correlations (H-7 to C-9 and C-6) permitted to locate the additional aromatic hydrogen at C-7.

Since amounts of compounds **2** and **3** were not enough to carry on a broad biological screening, three hemi-synthetic derivatives (**4-6**) of the natural *Penicillium* metabolites were prepared and tested.

All compounds were active on a preliminary disc diffusion assay against the bacteria *L. monocitogenes*, *S. pyogenes*, *Listeria sp.*, *S. typhimurium* and *B. cereus* but, due to availability restrictions, MIC were determined only for compound **1** and derivatives **4**, **5** and **6**. A screening for *Candida albicans* inhibition was also performed. Derivative **5** was not tested against *S. typhimurium* and *B. cereus* due to small amount available. Results are presented in Table 2.

Naphtalene derivatives are common as constituents of fungi (Frisvad et al. 1998; Vinokurova et al. 2005) and echinoderms (Bandaranayake, 2006). Conventional synthesis of naphthalenoids is a difficult task; therefore, isolation of compound **1** in the present study, with 45.34% yield from the extract (0.3175 mg.L<sup>-1</sup>) is very noteworthy making this compound a suitable starting compound for biotechnological use as a new drug lead, since its large scale production is feasible. The isolation of **1** in such high yield is also of great importance since this compound can easily be used as a marker for the identification of this *Penicillium* species. Yield improvement can still take place since modifications in the growing conditions can adequately improve the amount of synthesized secondary metabolites (Wijeratne et al. 2004; Nigam et al. 2007).

Cockrum et al. (1979) used weetbix, yeast extract, sucrose and Difco mycological broth for a comparative study between an Australian and a North American isolate of *Penicillium paxilli*. The medium used in the present work (glucose, peptone and yeast extract) has some similarities to the medium used by Cockrum et al. (1979). However paxilline, reported as a major metabolite of *P. paxilli* (Munday-Finch et al. 1996) was not detected in the present evaluation.

Narasimhachari and Vining (1963) reported the isolation of two red pigments from *P. herquei* and stated that, according to the growing substrate, this species can produce pigments of a variety of colors such as blue, yellow and green. The same authors described that, in a medium rich in peptone, as the one used for the current work, a deep blue pigment would be formed, fact also not observed in our experiments. Ding et al. (2008) have recently reported the isolation of a urea derivative, hualisyn from *P. herquei*, an aromatic compound that also is not related to the compounds related herein. Nevertheless producing secondary metabolites holding aromatic rings, metabolites previously described for *P. raistrickii* (Belofsky et al. 1998) are also not related to naphalenoids isolated in the present work.

The isolation and identification of compounds **1-3** and the fact that none of those were quoted in other previous studies of the closest species *P. paxilli*, *P. raistrickii* and *P. herquei* could indicate that we may have isolated a species still not described. However, since we obtained only one isolate of this fungus, no more conclusions can be drawn regarding its taxonomic placement. Efforts will be carried out in order to carry on DNA sequencing in order to unambiguously characterize this species.

In the bioassay, all compounds showed activity against all tested microorganisms, sometimes in concentrations comparable to the controls used, what is not very common since the controls are drugs currently in use, as an accomplishment of many steps of biotechnological improvement of production process. Minimum inhibitory concentration of **1** against *Candida albicans* (MIC 32 µg/mL) was the same as the activity showed by miconazole, the positive control. Noticeable were also the activities of **1** against *L. monocitogenes* and *B. cereus*, both assays resulting on a MIC value of 64 µg/mL. Acetylation of **1** led to a small decrease on its activity but the allylated and methoxylated derivatives (**5** and **6**) were much less active for all the tested strains. Compound **4** exhibited the same activity than positive control chloramphenicol (MIC 32 µg/mL) against *L. monocitogenes*, and presented the low MIC value of 64 µg/mL against *Candida albicans*.

While the acetatylated derivative exhibited good antimicrobial activities, the allyl and methoxy derivatives showed weaker ones, suggesting that the addition of an aliphatic chain lowers the activity. These results are corroborated by the greater bioactivity of **1**, which possesses three free hydroxyl groups conferring to it a higher polarity.

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