

AN ANTIMICROBIAL ALKALOID AND OTHER METABOLITES PRODUCED BY *Penicillium* sp. AN ENDOPHYTIC FUNGUS ISOLATED FROM *Mauritia flexuosa* L. f.

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The alkaloid glandicoline B (**1**) and six other compounds: ergosterol (**2**), brassicasterol (**3**), ergosterol peroxide (**4**), cerevisterol (**5**), mannitol (**6**) and 1-*O*- α -D-glucopyranoside (**7**) were isolated from *Penicillium* sp. strain PBR.2.2.2, a fungus from *Mauritia flexuosa* roots. The structures of the isolated metabolites were established by spectral analysis. MeOH extract of the fungal mycelium at 500 $\mu\text{g mL}^{-1}$ exhibited antimicrobial activity against *Staphylococcus aureus* and the compound **1** at 100 $\mu\text{g mL}^{-1}$ was active against *S. aureus*, *Micrococcus luteus* and *Escherichia coli*. The relationship between the bioactive properties of the fungus PBR.2.2.2 and those achieved for glandicoline B, as well the potential of this substance as bactericide is discussed.

Keywords: *Penicillium*; *Mauritia flexuosa*; glandicoline B.

INTRODUCTION

Plant-associated microorganisms are well known as great producers of natural products, being rich sources for biologically active metabolites with a wide-ranging application.¹ Their ability to use different solid substrates for their own metabolism, including vegetal sources, makes them a very interesting biochemical-chemical agent in nature, specially interfering in the host-plant development.² The host *Mauritia flexuosa* L. f. is a big palm tree from the Arecaceae family widespread by all South America.³ Its main constituents are flavonoids and triterpenoids.⁴ Plants of this species are used in the Amazonian folk-medicine, for burns and used as vermifuge.³ The fruits of this palm trees are also used to produce biofuels, cosmetics and as food by the local population.⁵

The *Penicillium* genus comprises more than 200 species, distributed through the entire world, being found in the most uncommon environments, such as the permafrost soil of the Arctic,⁶ some waste water from mines,⁷ and deep ocean sediments.⁸ Fungi from this genus are known as great producers of bioactive metabolites such as antimicrobial,⁹ immunosuppressants and cholesterol-lowering agents,¹⁰ anti-HIV¹¹ and antitumor drugs.⁸ Endophytes are a poorly investigated group of microorganisms that represent an abundant and dependable source of bioactive and chemically novel compounds with potential for exploitation in a wide variety of medical, agricultural, and industrial arenas.¹² In this context, the present work describes the isolation of some compounds from the *Penicillium* strain PBR.2.2.2 associated with the Amazonian palm tree *M. flexuosa* and their antibacterial activities.

RESULTS AND DISCUSSION

The EtOAc extract from the mycelial mass of the *Penicillium* sp. PBR.2.2.2 was chromatographed on silica gel columns and preparative

HPLC to give seven compounds (**1-7**) (Figure 1). The compounds **2-7** were identified as ergosterol,¹³ brassicasterol,¹⁴ ergosterol peroxide,¹⁵ cerevisterol,¹⁶ mannitol,¹⁷ and 1-*O*- α -D-glucopyranoside.¹⁸

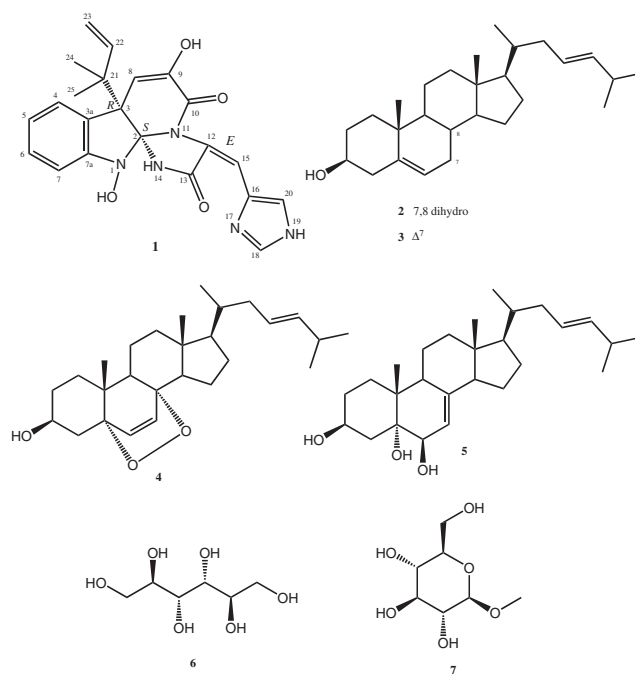


Figure 1. Structures from the isolated compounds 1-7

Compound **1** was obtained as a yellow gum. Its ¹³C NMR spectrum exhibited 22 signals which were assigned, by DEPT 135 and HSQC experiments to two methyls, one methylene, nine methines and ten quaternary carbons. Its APCI-MS spectrum contains an ion peak of [M+H]⁺ at *m/z* 420, consistent with the molecular formula C₂₂H₂₁N₅O₄ which also was in accordance with the NMR data. The

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identification of **1** as the indole alkaloid glandicolin B (Figures 2-3) was made sure as follow. Its ^1H NMR spectrum showed two duplets-like signals at δ_{H} 7.57 (H-4) and 6.96 (H-7) along with two triplets at δ_{H} 7.24 (H-6) and 7.01 (H-5), all signals with coupling constant of $J = 7.7$ Hz, characteristics of an *ortho* bi-substituted aromatic ring, which was confirmed by the COSY correlations of H-5 with H-4 and H-6, together with the correlation of H-6 and H-7. Besides, the HMBC correlations of these hydrogen atoms were useful to determine the six aromatic carbons and the C-3, at δ_{C} 54.3, which was detected in correlation with the H-4. This aromatic ring was achieved as part of the indole skeleton of **1** principally by the HMBC correlations among the signal at δ_{H} 5.37 (1H, s, H-8) with the C-3 as well with the signals at δ_{C} 103.5 (C-2), 127.8 (C-3a), 144.4 (C-9) and 161.0 (C-10). The isoprenyl unit could be verified by the HMBC correlations of hydrogen signals at δ_{H} 1.32 (3H, s, H-24), 1.29 (3H, s, H-25), 5.10 (1H, *dd*, $J = 10.8, 16.0$, H-23 *cis*) and 5.03 (1H, *dd*, $J = 16.0, 17.2$, H-23 *trans*) with the carbon signal at δ_{C} 43.6 (C-21) as well by the COSY correlations of both H-23 one each other and with the methine hydrogen at δ_{H} 6.22 (1H, *dd*, $J = 10.8, 17.2$, H-22). The H-24 and H-25 also have HMBC correlation with the C-3 ratifying this carbon as the connection point of the isoprenyl group of **1**. Finally the histidine group was indicated by the deshielded signals at δ_{H} 8.26 (1H, s, H-15), 7.90 (1H, s, H-18) and 7.37 (1H, s, H-20) and their HMBC correlations: H-15 with carbon signals at δ_{C} 167.2 (C-13), 131.4 (C-20) and 127.4 (C-12), H-18 with a signal at δ_{C} 128.8 (C-16) and the C-20, and H-20 with a signal at δ_{C} 137.6 (C-18) and the C-16. While no correlation could be observed between the histidine moiety and the remainder of **1**, the chemical shifts of C-12, C-13 and C-2, all non-hydrogenated carbons, are in coherence with the known structure of glandicolin B. Since this substance is the direct precursor to meleagrín which is the *O*-methylated version of the glandicoline B, its absolute configurations on the double bond between C-12 and C-15 and on the C-2 and C-3 were deduced as *E*, *S* and *R*, respectively, as established by crystallography for meleagrín.¹⁹ All NMR data obtained for **1** are in the Table 1.

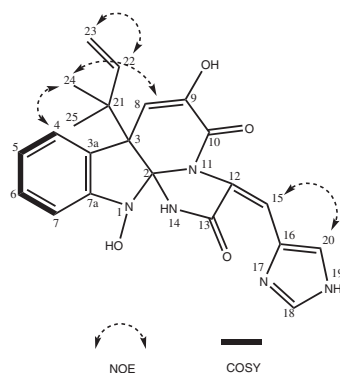


Figure 2. ^1H - ^1H NOE and COSY correlations for the compound **1**

The CID spectra of the product ions of the peak at m/z 420 at 15 eV also confirmed **1**, mainly by the fragments at m/z 351 and 334 corresponding to the losses of the isoprenyl and hydroxyl groups respectively, the ion 289 corresponds to a formamide loss from m/z 334. The ions at m/z 261 and 233 are derived from CO losses from the ion at m/z 289.

Isolation of Glandicoline B has been published only once previously, from a soil strain of *Penicillium glandicola*,²⁰ and its identification was done by comparison between its ^1H NMR (acetone- d_6), IR, UV and MS (EI) spectra and meleagrín data. All NMR (methanol- d_4) and MS (APCI) data of **1** are published for the first time for glandicoline B. This substance is an important intermediate in the

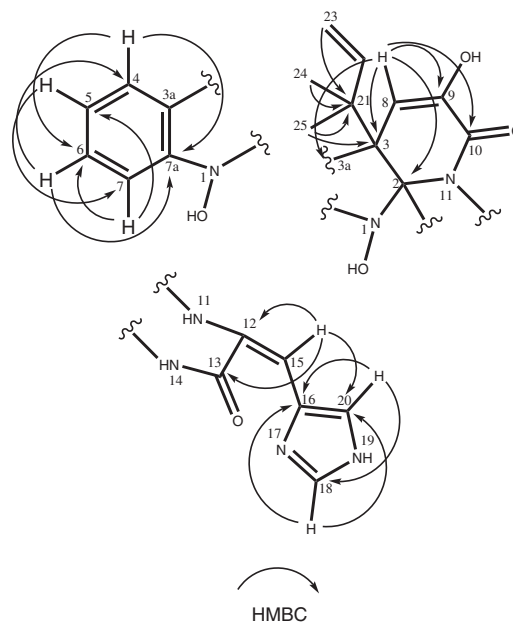


Figure 3. ^1H - ^{13}C HMBC correlations for the compound **1**

Table 1. Spectral data of compound **1**, δ in ppm (^1H , 500 MHz; ^{13}C , 125 MHz)

	δ_{C}	δ_{H} , mult, (J in Hz)	COSY	HMBC	NOE
1	-				
2	103.5				
3	54.3				
3a	127.8				
4	125.8	7.57, d, (7.7)	H5	C3, C6, C7a	H24
5	123.7	7.01, t, (7.7)	H4, H6	C3a, C7	
6	129.4	7.24, t, (7.7)	H5, H7	C4, C7a	
7	113.1	6.96, d, (7.7)	H6	C3a, C5	
7a	149.8				
8	110.8	5.37, s		C2, C3, C3a, C9, C10	H23, H24
9	144.4				
10	161.0				
11	-				
12	127.4				
13	167.4				
14	-				
15	108.2	8.26, s		C12, C13, C20	H20
16	128.8				
17	-				
18	137.6	7.90, s		C16, C20	
19	-				
20	131.4	7.37, s		C16, C18	H15
21	43.6				
22	146.7	6.22, dd, (10.8; 17.2)			
23a	113.7	5.10, dd, (10.8; 16)		C21	
23b	113.7	5.03, dd, (17.2; 16)		C21	
24	n.d.	1.32, s		C3, C21	H4, H8
25	30.9	1.29, s		C3, C21	

biosynthesis of the mycotoxins meleagrins and oxalins,²¹ which are produced by some *Penicillium* species, as well the N-hydroxylated form of the glandicoline A, which is derived from some rearrangements reactions of the diketopiperazine alkaloid roquefortine C, another mycotoxin with neurotropic and antibiotic activities, commonly found in *Penicillium* spp. cultivation.²²

Biological assay

The mycelial extract in methanol of the *Penicillium* sp. strain PBR.2.2.2 exhibited antimicrobial activity at 500 µg mL⁻¹ against *Staphylococcus aureus* and compound **1** was active at 100 µg mL⁻¹ against *S. aureus*, *Micrococcus luteus* and *Escherichia coli*. The other six compounds did not show activity against those bacteria. The glandicoline B exhibited a low bactericidal activity if compared with the positive controls norflaxacin and tetracycline. While other compounds of the roquefortine/oxaline biosynthesis²² have been shown important biological activities, e. g. the cytotoxicity of some diterpenoid derivatives of meleagrins⁸ and the bactericidal activity of the roquefortines,²³ there is no previous report about biological activities for glandicoline B.

EXPERIMENTAL

Instrumental and chromatographic materials

The optical rotation was measured in MeOH using a Perkin Elmer[®] 241 polarimeter with a sodium lamp at 598 nm at 33 °C. 1D and 2D NMR experiments were obtained in CD₃OD and CDCl₃ (Cambridge Isotope[®]) using a Varian[®] INOVA 500 spectrometer operating at 500 MHz for ¹H and 125 MHz for ¹³C. TMS was used as internal standard. Spectra of APCI-IT-MS were obtained in an LCQ fleet equipment (Thermo[®]) operating in positive mode. The analysis of product ions until MS⁴ was performed by collision-induced dissociation (CID) with energy of 15 eV. TLC was performed using silica gel 60 PF254 (Merck[®]) and spots were visualized under UV light and sprayed vanillin-H₂SO₄ reagent, followed by heating at 100 °C. The fractionation was performed in CC using normal phase silica 230-400 mesh (Merck[®]). Analytical HPLC was performed on a Shimadzu SPD-M10AVP diode array detector, LC-10AD pumps, SIL-10ADVP auto sampler and UV-vis SPD-6A detector. Preparative HPLC: Shimadzu UV detector LC-6AD pump, UV SPD-10AV detector and manual injector. Analytical HPLC column: Phenomenex, Luna, Phenyl-Hexyl (5 µm, 250 mm x 4.60 mm); preparative HPLC column: Phenomenex, Luna, Phenyl-Hexyl (5 µm, 250 mm x 21.20 mm).

Isolation and identification of *Penicillium* sp. PBR.2.2.2

Roots of *M. flexuosa* were collected in June 2008 in the campus of the Universidade Federal do Amazonas (UFAM) in the city of Manaus, Brazil, and authenticated by the Herbarium of the UFAM where a voucher specimen (no. 7282) is deposited. The plant material was washed with detergent and sterile water for external cleaning, then fragments were immersed in 70% alcohol, later in 3% hypochlorite solution and finally in sterile water.¹² After this process, plant fragments were inoculated in Petri dishes containing ISP2-agar medium and incubated up to thirty days. At 24-h intervals, after the third day, fragments of agar containing hyphae of the newly born endophytic fungi were removed and transferred to test tubes with the same medium and incubated at 26 °C for 30 days. After this period the mitospore fungi were purified by the Tween technique.¹² The identification of the isolated endophytic fungus *Penicillium* sp. PBR.2.2.2 was conducted by microculture of it in different solid media: PDA (potato, dextrose and agar), ISP2 (international *Streptomyces* project

number 2) and Czapek-agar. The vegetative and reproductive morphological structures observed in the slides of the culture with 24 and 48 h in optical microscope were compared with the literature.²⁴ A voucher was deposited in the fungal collection of the GEMMA group of the UFAM.

Cultivation and extraction

Penicillium sp. strain PBR.2.2.2 was grown on PDA culture medium at 26 °C for 8 days. After this period a suspension of its cells was prepared, quantified using McFarland scale and inoculated (50 µL) in 60 flasks of 1 L containing 300 mL of ISP2 liquid medium (10 g corn starch, 4 g yeast extract, 10 g malt, 4 g dextrose for every 1 L of distilled water). The cultivation was made at 26 °C in static mode for 21 days. The mycelial mass (1.7 kg) was ground and extracted with methanol (3 x 1 L) providing 10.5 g of crude extract.

Isolation of the chemical constituents

The methanolic extract was submitted to liquid vacuum chromatography on silica gel CC (70-230 mesh). A gradient system elution was applied (hexane:EtOAc 9:1 and 3:7, EtOAc 100% and MeOH 100%) resulting in 4 fractions which were monitored by silica gel TLC. The both hexane:EtOAc fractions were rechromatographed on a low-pressure silica gel CC (230-400 mesh) (h x Ø = 40 x 2 cm) eluted with dichloromethane, ethyl acetate and methanol gradients affording ergosterol **2** (DCM 100%, 13.4 mg), brassicasterol **3** (DCM:EtOAc 95:5, 16.2 mg), ergosterol peroxide **4** (DCM:EtOAc 85:15, 34.7 mg), cerevisterol **5** (DCM:EtOAc 1:1, 4.8 mg). The EtOAc 100% fraction was analysed in analytical HPLC. Isocratic elution with 50% MeOH at a flow rate of 0.7 mL min⁻¹ gave the best chromatographic resolution. According to the scaling up from analytical HPLC conditions, the EtOAc 100% fraction was submitted to reverse phase preparative HPLC, with 50% MeOH at 21.3 mL min⁻¹ and UV detection at 245 nm, to afford **1** (23.5 mg). The MeOH 100% fraction when rechromatographed in a low-pressure silica gel CC (230-400 mesh) (h x Ø = 30 x 1.5 cm) eluted with dichloromethane, ethyl acetate and methanol gradient afforded mannitol **6** (12.9 mg) and 1-*O*- α -D-glucopyranoside **7** (4.2 mg).

Glandicoline B (**1**): yellow gum; $[\alpha]_D + 23.8$ (c 0.14 in MeOH); ¹H-NMR (500 MHz, CD₃OD), ¹³C (125 MHz), COSY and HMBC: Table 1; APCI-MS daughter ions (15 eV): *m/z* 420 (5) ([M+H]⁺, 403 (5), 374 (2), 351 (10), 334 (20), 316 (10), 289 (100).

Antimicrobial assay

The minimal inhibitory concentrations (MICs) were determined by microbroth dilution assays as recommended by the Subcommittee on Antifungal Susceptibility Testing of the US National Committee for Clinical Laboratory Standards (NCCLS). The assays were performed on 96 well plates with 100 µL of Mueller Hinton Broth (MHB), 100 µL of test compound or fraction solutions and 5 µL of test bacterial suspensions at 1.0 x 10⁷ UFC mL⁻¹. The fungus compounds or fractions assayed were dissolved in dimethylsulfoxide (DMSO) at initial concentration of 500 µg mL⁻¹ and the incubation was made at 37 °C for 24 h. The microorganisms tested were *E. coli*, *S. aureus*, *Pseudomonas aeruginosa*, *M. luteus* and *Bacillus cereus*. The bioactivities were recorded as blue coloration in the wells after use of resazurin dye. The bacteriostatic or bactericidal effects of the metabolites assayed were observed by inoculation of the well materials on Mueller Hinton Agar plates after the tests. Positive controls norflaxacin (4 µg mL⁻¹) and tetracycline (4 µg mL⁻¹) with the negative control DMSO were used during the tests.

CONCLUSIONS

Penicillium sp. strain PBR.2.2.2 is the first reported endophytic fungus from the palm tree *M. flexuosa*. From its bioactive mycelium extract were isolated seven compounds, including the rare indole alkaloid glandicoline B, which exhibited moderate antimicrobial activities against *S. aureus*, *M. luteus* and *E. coli*. This alkaloid, an intermediate in the biosynthesis of the roquefortine/oxaline pathway,²² was isolated previously only once, from *Penicillium glandicola*, a species found in Russian permafrost soil,²⁰ a totally different environment from that from where obtained the *Penicillium* sp. strain PBR.2.2.2. All NMR and MS data of glandicoline B, new in the literature, confirm the structure previously described.²⁰ The bioactive properties of the fungus PBR.2.2.2 can be attributed, at least in part, to glandicoline B, the major component of its mycelium and a potential agent for bactericide application.

SUPPLEMENTARY MATERIAL

The NMR ¹H, HMBC, HSQC, COSY, NOE, NMR ¹³C and MS spectra of **1** are available with free access in a pdf file, at <http://quimicanova.sbq.org.br>.

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