The Pharmaceutical and Chemical Journal, 2019, 6(3):1-5

Available online www.tpcj.org

Research Article

ISSN: 2349-7092
CODEN(USA): PCJHBA

A Phenolic Glycoside from *Beauveria bassiana* ILB 204 Fermentation Broth

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Abstract In spite of the great advances in chemotherapeutics, infectious diseases are still one of the leading causes of death in the world. Thus, development of new antimicrobial compounds against different microorganisms and with new mechanisms of action is becoming critically important. In our continuous search for new antimicrobial molecules we screened the culture broth of several soil microorganisms. A *Beauveria* isolate showed promising activity and we isolated and determined the structure of a phenolic glycoside (6-Methyl-1,2,4-trihydroxybenzene-1-*O*-β-D-4´-methyl-glucopyranoside)never before reported in this species.

Keywords Soil microorganism, antimicrobial

Introduction

Beauveria is a cosmopolitan anamorphic genus of arthropod pathogens that include the agronomically important species *B. brongniartii*, and especially *B. bassiana*, which are used as mycoinsecticides [1-2] for the biological control of pest insects [3]. *Beauveria bassiana* is perhaps the most extensively studied and used entomopathogenic fungus worldwide, mainly due to its wide host range, which includes more than 700 species of insects [4,5].

The genus *Beauveria* is also known to produce different toxins and enzymes. In the cell-free filtrates of *Beauveria bassiana* cultures, several secondary metabolites, as beuvericin, bassilalone and pyridovericin [6-8] were found, although most of the work has been done on the notorious red pigment oosporein [9-12]. In a joint project with the National Institute for Agricultural Research (INIA) we screened a large number of fungal strains for antimicrobial activity.

In this work we present the isolation and structural elucidation of a phenolic glycoside from de culture broth of *B. bassiana* ILB 204 as well as its antimicrobial activity.

Materials and Methods

Fungal strain

Isolate ILB 204 of the INIA Las Brujas collection was originally obtained from a *Eurymetopus fallax* insect and identified as *B. bassiana* based on morphological features. For the molecular identification, DNA was obtained from mycelium grown on Potato Dextrose Agar plate using the DNeasy Plant Mini Kit (Qiagen). Genomic DNA was used as template for the PCR amplification of part of the gene that codes for the elongation factor (EF1α) with primers EF1–728F and EF1–986R [13]. The PCR product was sequenced in both directions with the same primers by Macrogen (Korea). Sequences were corrected manually and aligned using MEGA6.06 [14] together with

sequences of different *Beauveria* species. The alignment was used to perform a phylogenetic analysis by Maximum Likelihood with MEGA and Bootstrap 1000. The sequence was submitted to GenBank with accession number MH507144. Sequence codes and strain denominations are presented in Fig. 1.

Fermentation Conditions

Isolate ILB 204 was grown on a PDA platefor 10 days, when sporulation was observed. Spores were collected with 5 ml of Tween-20 water with the help of a sterilized glass bar. The number of spores per mL was determined by counting in a hemocytometer. An aliquot of 900 ul of the $4.5x10^5$ spores/mL suspension was used to inoculate each of 5Erlenmeher flasks of 250 mL containing 120 mL of broth to reach an initial concentration of $3.4x10^3$ spores/mL. The liquid culture medium comprised 30 g of sucrose, 10 g bactopeptone (Oxoid), 1 g K_2HPO_4, 0.5 g KCl and 1 g $MgSO_4.7H_2O$ in 1 Lt of distilled water. The flasks were incubated in an orbital shaker at 25 °C and 180 RPM for 96 hs when the broth was collected and vacuum filtered through a Wathman2 filter paper to retain the fungal mass and obtain the mycelium-free filtrate. The filtrate was supplemented with chloroform at a ratio of 1 ml every 100 ml and kept in the fridge until further processing.

Bacterial Strains

Test organism used were *Acinetobacter baumanii* CCMGb1, *Enterobacter cloacae* CCMGb3, *Enterococcus faecalis* CCMGb2, *Klebsiella pneumoniae* CCMG12716, *Pseudomonas aeruginosa* ATCC15442, *Staphylococcus aureus* ATCC6538and *Escherichia coli* ATCC25922.

Antibiotic Susceptibility Assay

Minimum inhibitory concentrations (MICs) were determined as the lowest concentration of each antibiotic at which there was no visible growth. MICs were determined using the microdilution technique according to the Clinical and Laboratory Standards Institute protocols [15].

Bioautography used thin-layer chromatography (TLC) plates according to the agar overlay method described by Rahalison et al [16] using *S. aureus* ATCC6538p.

Extraction and Isolation

Spent broth (850 mL) was saturated with NaCl, filtered and extracted with AcOEt (300 mLx3). The extract was then dried and evaporated under vacuum to obtain a dark red oil (180 mg).

Part of the extract was submitted to vacuum liquid chromatography (flash SiGel, Machery Nagel) and eluted with CH_2Cl_2/MeOH solutions of increasing polarity.

The MeOH fraction was further purified using preparative TLC (pTLC) in the same system to obtain an enriched fraction and then preparative TLC using CH_2Cl_2/MeOH 80:20 as developing solvent affording a pure compound (4 mg).

Structural Analysis

A BruckermicrOTOF Q-TOF with ESI source in positive mode for HR-MS spectra and a Shimadzu QP 5050 for direct infusion MS were used.

TLC was performed on silicagel (MachereyNagel, Dürin, Germany) using $CHCl_3$/MeOH (80:20) as development solvent and H_2SO_4/heating or Fast Blue B salt as detection reagents.

^1H NMR and ^{13}C NMR spectra were obtained at 400 MHz and 100 MHz respectively, on a BruckerAvance DPX 400 spectrometer, using $CDCl_3$ as solvent and TMS (δH 0.00) and acetone (δC 31.00) as references. 2D (different H-H COSY, HMBC, HSQC) experiments were carried out with programs available in the Brucker software.

UV-vis spectroscopy was performed using a Shimadzu UV 1800 spectrophotometer.

Results and Discussion

The phylogenetic analysis produced a tree in which ILB 204 clustered with isolates of *B. bassiana* with 99 % bootstrap support, which confirmed the identification based on morphological features (Fig. 1).

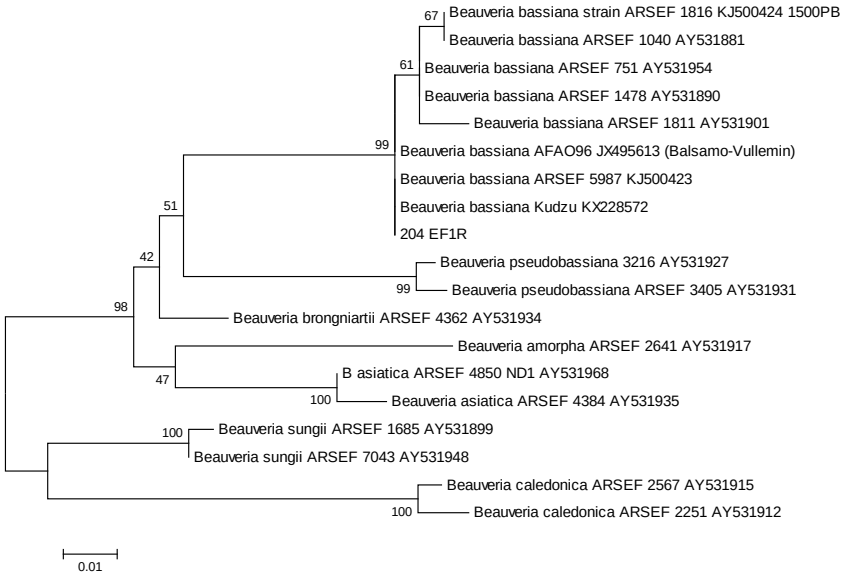

Figure 1: Phylogenetic tree produced by Maximum likelihood method of sequence of ILB 204 and sequences retrieved from GenBank. Bootstrap support is shown on the nodes

The AcOEt extraction of the fermentation broth gave an oily residue that after VLC and preparative TLC afforded a pure compound.

The UV-Vis spectra presented a maximum at λ=279 nm characteristic of a substituted aromatic ring.

The HR-MS showed a quasimolecular peak at 317.1234 $[M+H]^+$ (calc. $C_{14}H_{20}O_8$ 316.1236) and signals at 316 $[M]^+$and 140 $[C_7H_8O_3]^+$ in the EI-MS.

In the ^{1}H-NMR spectrum among the several signals observed especially explanatory were the ones at δ=4.43 (J= 7Hz) characteristic of an anomeric proton, d=3.58 (s, 3H) indicative of methoxy group and aromatic protons at d=6.11 and 6.16.

In the ^{13}C-NMR experiments 14 signals could be seen. Again the signal at δ=106.5 is compatible with a anomeric carbon and critical correlations found in COSY, HSQC and HMBC spectrum are shown in Figure 2.

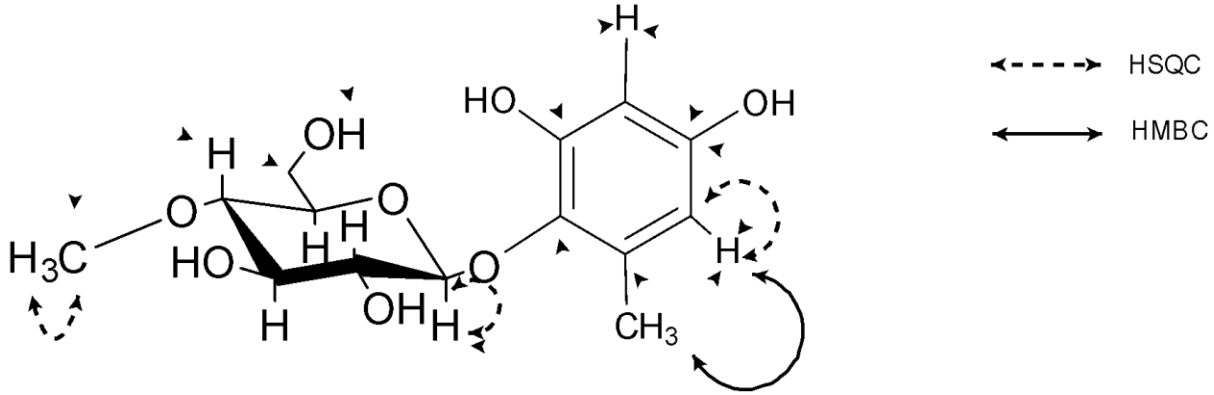

Figure 2: Critical correlations and structure of compound 1

Through 2-D experiments all signals could be assigned (Table 1) and thus the compound was identified as 6-Methyl-1,2,4-trihydroxybenzene-1-*O*-β-D-4´-methyl-glucopyranoside. This compound was never reported for *B. bassiana* before.

Table 1: Chemical shift data for compound 1

	C (δ ppm)	H (δ ppm, m, *J* Hz)
1	137.5	--
2	--	--
3	100.4	6.16, d, 1H, 3.0Hz
4	--	--
5	107.7	6.11, d, 1H, 3.0Hz
6	131.5	--
7	15.7	2.27, s, 3H
1´	106.5	4.43, d, 1H, *7.5*Hz
2´	74.2	3.49, dm, 1H , *7.5*Hz
3´	76.5	3.50 m, 1H
4´	78.7	3.25, d, 1H *9.5*Hz
5´	75.9	2.26, m, 1H
6´	60.7	3.72/3.82 , dm, 2H, *11*Hz
OCH_3	59.4	3.58, s, 3H

The antimicrobial activity of the extract was tested against a panel of bacteria of the ESKAPE group showing low activity (2500-1250 μg/mL) for *K. pneumoniae*, *E. faecalis*, *E. cloacae*, and *A. baumanii* and medium activity (<625 μg/mL) for *E. coli*, *Ps. Aeruginosa*, and *S. aureus.*

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