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Short communication

Two cytotoxic triterpenes from cultures of a Kenyan *Laetiporus* sp. (Basidiomycota)

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ABSTRACT

HPLC profiling of the mycelial culture of a poroid basidomycete collected in Mount Elgon, Kenya, which probably represents a new species of the genus *Laetiporus*, led to isolation of two previously undescribed lanostane type triterpenes.We propose the trivial names laetiporins A (**1**) and B (**2**). In addition, five known ones: dehydrosulphurenic acid (**3**), sulphurenic acid (**4**), eburicoic acid (**5**), 15α-hydroxytrametenolic acid (**6**) and trametenolic acid (**7**) were also isolated. The laetiporins (**1–2**) exhibited significant cytotoxic effects against various human cancer cells. The known compounds (**3–5**) and (**7**) also showed moderate cytotoxic activity, but none of the compounds showed any significant antimicrobial activity.

1. Introduction

Exploitation of natural resources in particular fungi in search of new bioactive compounds has been an emerging field of study. Basidiomycetes seem greatly promising as sources for new, interesting classes of compounds of different biogenetic origins with various biological activities (De Silva et al., 2013; Richter et al., 2015). Even though the tropics are the home of most of the basidiomycetes species, its riches remain largely untapped. Previously we reported the isolation of several new bioactive compounds from the Kenyan basidiomycetes *Favolaschia calocera* and *Laxitextum incrustatum* (Chepkirui et al., 2016; Mudalungu et al., 2015).

In our present study, several strains of a *Laetiporus* species (Polyporaceae) originating from Kenya were investigated. *Laetiporus* is one of the few polypore genera that produce conspicuous basidiomes that are edible when young, such as *L. sulphureus*, the common well-known "chicken of the woods". The genus has a world-wide distribution. *Laetiporus* spp. also have been recognized as important forest pathogens, causing a cubical brown rot in living and dead wood of both conifers and angiosperms (Banik et al., 2010).

2. Experimental section

2.1. General experimental procedure

Optical rotations were determined with a Perkin-Elmer 241 spectrometer; UV spectra were recorded with a Shimadzu UV–vis spectrophotometer UV-2450. NMR spectra were recorded with Bruker Ascend 700 spectrometer with 5 mm TXI cryoprobe (^{1}H 700 MHz, ^{13}C 175 MHz) and Bruker AV II-600 (^{1}H 600 MHz, ^{13}C 150 MHz) spectrometers. HR-ESI–MS mass spectra were recorded with Agilent 1200 series HPLC-UV system (column 2.1 × 50 mm, 1.7 µm, C18 Acquity UPLC BEH (waters), solvent A: H_2O + 0.1% formic acid; solvent B: AcCN + 0.1% formic acid, gradient: 5% B for 0.5 min increasing to 100% B in 19.5 min and then maintaining 100% B for 5 min, flow rate 0.6 mL/min^{-1}, uv/vis detection 200–600 nm combined with ESI-TOF-MS (Maxis, Bruker) [scan range 100–2500 *m/z*, capillary voltage 4500 V, dry temperature 200 °C].

2.2. Fungal material

The fungal strain *Laetiporus* sp. was collected from Kakamega equatorial rainforest, located in the western part of Kenya (0°17′3.19" N 34°45′8.24" E) on a dead fallen trunk of *Bridelia micrantha* (Phyllanthaceae) by C. Decock, Feb. 17, 2015 (collection

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and isolation number KE-15-22). The dried herbarium specimen and culture are deposited at MUCL, Louvain-la-Neuve, Belgium as MUCL 55532. The fungus was assigned to the genus *Laetiporus* by morphological studies and sequencing of the rDNA (5.8S gene region, the internal transcribed spacer ITS1 and ITS2). This fungus probably represents a new taxon and is presently being included in a large study on the phylogeny and taxonomy of the genus *Laetiporus* in Africa.

Genomic DNA Miniprep kit (Bio Basic Canada Inc., Markham, Ontario, Canada). A Precellys 24 homogenizer (Bertin Technologies, France) was used for cell disruption at a speed of 6000 rpm for 2 × 40 s. The gene regions were amplified with primers ITS 1f and NL4.

2.3. Small scale fermentation

Laetiporus sp. strain MUCL 55532 was cultivated in three different liquid media YMG, Q6 ½ and ZM ½ media (for details on the composition of these media see Supplementary information). These three media were selected because previous studies had revealed that they were optimal for attaining complementary secondary metabolites profiles in filamentous fungi (Bitzer et al., 2008). A well grown culture grown on an YMG agar plate was cut into small pieces using a cork borer (7 mm) and five pieces inoculated in a batch of ten 500 mL Erlenmeyer flask containing 200 mL of the three media. The cultures were incubated at 23 °C on a rotary shaker (140 rpm). The growth of the fungus was monitored by constantly checking the amount of free glucose (using Bayer Diastix Harnzuckerstreifen). The fermentation was terminated two days after glucose depletion (Bitzer et al., 2008).

2.4. Extraction of the small scale fermentation crude extracts

The supernatant and the mycelia from the small scale fermentation were separated by filtration. The supernatant was extracted with equal amount of ethyl acetate and filtered through anhydrous sodium sulphate. The resulting ethyl acetate extract was evaporated to dryness by means of rotary evaporator. The mycelia were extracted with 200 mL of acetone in ultrasonic bath for 30 min, filtered and the filtrate evaporated. The remaining water phase was suspended in equal amount of distilled water and subjected to same procedure as the supernatant. The mycelia and supernatant crude extracts from the three media HRMS were measured. Analysis of the MS spectra by comparing the masses of the detected peaks and their molecular formula obtained from HRMS with those in the data base (Dictionary of natural products) led to the identification of the new compounds on the YMG mycelia crude extract (Dictionary of Natural Products on DVD, 2016).

2.5. Large scale fermentation

A well-grown seven days old YMG agar plate of the mycelial culture was cut into small pieces using a 7 mm cork borer and five pieces inoculated in 1000 mL Erlenmeyer flask containing 500 mL (10 flasks) of YMG medium. The culture was incubated at 23 °C on a rotary shaker (140 rpm) for 36 days. Fermentation was aborted 2 days after the depletion of free glucose.

2.6. Extraction of the large scale fermentation crude extracts

The mycelia and supernatant from the large scale fermentation were separated via vacuum filtration. The mycelia were extracted with 4 × 500 mL of acetone in ultrasonic bath for 30 min. The extracts were combined and the solvent evaporated by means of a rotary evaporator. The remaining water phase was subjected to the same procedure as mycelium in small scale extraction three times yielding white solid (1.2 g). The supernatant on the other hand was mixed with 100 g adsorbent resin (Amberlite XAD™–16 N) and incubated overnight on a shaker. The Amberlite resin was then filtered and eluted with 4 × 500 mL acetone. The resulting acetone extract was evaporated and the remaining water phase subjected to the same procedure as the acetone water phase of the mycelia to afford 250 mg dark brown solid.

2.7. Isolation and purification

The mycelial crude extract was fractionated using preparative reverse phase liquid chromatography (PLC 2020, Gilson, Miiddleton, USA). VP Nucleodur 100-5C 18 ec column (250 × 40 mm, 7 µm: Macherey-Nagel) used as stationary phase. Deionized water (Milli-Q, Millipore, Schwalbach, Germany) (solvent A) and acetonitrile (solvent B) were used as the mobile phase. The elution gradient used was 5-100% solvent B in 60 min and thereafter isocratic condition at 100% solvent B for 5 min. UV detection was carried out at 210, 254 and 350 nm. Ten fractions were collected according to the observed peaks (F-1–F-10) for mycelia extract. For details on the purification of fractions see SI.

2.8. Antimicrobial assay

The antifungal and antibacterial activities Minimum Inhibition Concentrations (MIC) were determined in serial dilution assay as described previously (Halecker et al., 2014; Wittstein et al., 2016) against *Candida tenuis* MUCL 29982, *Mucor plumbeus* MUCL 49355, *Escherichia coli* DSM498 and *Bacillus subtilis* DSM10. The assays were carried out in 96-well microtiter plates in YMG media for filamentous fungi and yeast and EBS for bacteria. For the small scale fermentation extracts and pure compounds the starting concentration was 300 µg/mL.

2.9. Cytotoxicity assays

In vitro cytotoxicity (IC50) of compound **1-7** was determined against a panel of mammalian cell lines including mouse fibroblast L929, HeLa (KB-3-1), epidermoid carcinoma cells A431, breast cancer cells MCF-7, prostate cancer cells PC-3 and adenocarcinomic human alveolar basal epithelial cells A549. The cell lines L929, KB 3.1and A549 were cultured in DMEM (Gibco), PC-3 in F12-K (Gibco), MCF-7 and A431 in RPMI (Gibco) media, all supplemented with 10% of fetal bovine serum (Gibco) under 10% CO_2 at 37 °C. The cytotoxicity assay was performed according to the MTT (3-(4,5-dimethylthiazol-2- yl)-2,5 diphenyltetrazolium bromide) method in 96-well microplates (Mosmann, 1983). Briefly 60 µL aliquots of serial dilutions from an initial stock of 1 mg/mL in MeOH of the test compounds were added to 120 µL aliquots of a cell suspension (5.0×10^4 cells/mL) in 96-well microplates. After 5 days incubation, a MTT assay was performed, and the absorbance measured at 590 nm using an ELISA plate reader (Victor). The concentration at which the growth of cells was inhibited to 50% of the control (IC_{50}) was obtained from the dose response curves. Negative control was methanol.

3. Results and discussion

Crude extract fractionation and subsequent purification by reverse phase HPLC led to the isolation of two previously undescribed metabolites, for which we propose the trivial names, Laetiporin A (**1**) and B (**2**), together with the five known compounds: dehydrosulphurenic acid (**3**) (Wu et al., 2005), sulphurenic acid (**4**) (Fried et al., 1964), eburicoic acid (**5**) (Kariyone and Kurono, 1940), 15α-hydroxytrametenolic acid (**6**) (Yang et al., 1996) and trametenolic acid (**7**) (Yoshikawa et al., 2000) (Fig. 1). The known compounds were identified by comparing their NMR and HR-MS data with those reported in the literature.

Compound **1** was isolated as a white solid with the molecular formula $C_{31}H_{48}O_3$ and 8 levels of unsaturation established from HRMS. Peaks at *m/z* 469.3682 for $[M+H]^+$ and *m/z* 451.3572 for $[M+H-H_2O]^+$ were further observed in the HRMS. ^{13}C NMR spectroscopic