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An unprecedented spiro [furan-2,1'-indene]-3-one derivative and other nematicidal and antimicrobial metabolites from *Sanghuangporus* sp. (Hymenochaetaceae, Basidiomycota) collected in Kenya

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ABSTRACT

Bioassay guided fractionation of extracts derived from submerged cultures of a *Sanghuangporus* sp. (i.e., the genus that was until recently referred to as the "*Inonotus linteus* complex" of medicinal mushrooms) originating from Kenya led to the isolation of a new spiro [furan-2,1'-indine]-3-one derivative, for which we propose the trivial name phelligridin L (**1**) together with the known compounds 3,14′-bihispidinyl (**2**), hispidin (**3**), ionylideneacetic acid (**4**), 1*S*-(2*E*)-5-[(1*R*)-2,2-dimethyl-6-methylidenecyclohexyl]-3-methylpent-2-enoic acid (**5**), phellidine E (**6**) and phellidine D (**7**). Compounds **1–3**, showed moderate nematicidal activity against *Caenorhabditis elegans* with LD_{50} of 12.5 µg/m. The nematicidal activity of 3, 14′-bihispidinyl and hispidin (**1**, **2**) has not been reported before. Furthermore, compounds **1–5** demonstrated moderate antimicrobial activity against various test organisms.

1. Introduction

Fungi have been recognized as source of structurally unique and bioactive metabolites (Karwehl and Stadler, 2016). The fungal diversity in the tropical rainforest ecosystems has been underexplored, since the majority of the fungal species found in these habitats have not been described nor has their chemistry been studied. In an effort to document this diversity and its rich chemistry, we embarked on extensive study of the secondary metabolite production of several basidiomycetes collected from Kenya's tropical rain forest Kakamega. Novel structurally diverse and bioactive metabolites like laetiporins, calocerins, 9-oxostrobilurins, laxitextines, microporenic acids and aethiopinolones are some of the metabolites that we have reported recently (Chepkirui et al., 2018a, 2018b, 2017, 2016; Mudalungu et al., 2015) from our ongoing study.

The present paper deals with a species belonging to the genus *Sanghuangporus* (Hymenochaetaceae), whose extracts from mycelial cultures had shown prominent antimicrobial effects during the course of the aforementioned study. The specimen was collected from the Kakamega Nature Reserve, a spot of rain forest at medium elevation in Kenya. The genus *Sanghuangporus* was erected for the "*Inonotus linteus* complex", and several additional species from the Paleotropics previously included in *Phellinus* (Zhou et al., 2015). It originally included *S. alpinus, S. baumii, S. lonicericola, S. lonicerinus, S. microcystideus, S. sanghuang, S. vaninii, S. weigelae, S. weirianus* and *S. zonatus* (Zhou et al., 2015). Three more species have been added later, *viz. S. ligneus, S. pilatii* and *S. quercicola* (Ghobad-Nejhad, 2015; Tomsovsky, 2015; Zhu et al., 2017). The Asian species have been referred to in the literature as medicinal mushrooms. Only one species in the genus, *S. microcystideus*, has been reported from Eastern Africa (Zhou et al., 2015). In this study we report the isolation, structure elucidation and biological activities of secondary metabolites from another African *Sanghuangporus* sp., which probably represents an undescribed species.

2. Experimental section

2.1. General experimental procedure

Optical rotations were determined with a Perkin-Elmer (Überlingen, Germany) 241 spectrometer; UV spectra were recorded with a Shimadzu (Duisburg, Germany) UV–vis spectrophotometer UV-2450. NMR spectra were recorded with a Bruker (Bremen, Germany) Ascend

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700 spectrometer with 5 mm TXI cryoprobe (^{1}H 700 MHz, ^{13}C 175 MHz) and Bruker AV II-600 (^{1}H 500 MHz, ^{13}C 150 MHz) spectrometers. HR-ESI–MS mass spectra were recorded with a Bruker (Bremen, Germany) Agilent 1200 series HPLC-UV system (column 2.1 × 50 mm, 1.7 µm, C18 Waters Acquity UPLC BEH, solvent A: H2O + 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]. Chemicals and solvents were obtained from AppliChem GmbH, Avantor Performance Materials, Carl Roth GmbH & Co. KG and Merck KGaA in analytical and HPLC grade.

2.2. Fungal material

The specimen MUCL 55592 was collected from Kakamega equatorial rainforest, located in the western part of Kenya (0°17′3.19"N 34°45′8.24"E) by C. Decock on Feb. 17, 2015. The dried herbarium specimen and culture are deposited at MUCL, Louvain-la-Neuve, Belgium (designation no. MUCL 55592). The fungus was identified as a species of the genus *Sanghuangporus* by morphological examination and sequencing of the rDNA (5.8S gene region, the internal transcribed spacer (ITS) and part of the nuclear ribosomal large subunit (nLSU).

DNA extraction was performed as reported previously (Wendt et al., 2018) with the EZ-10 Spin Column 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 for sequencing of the rDNA (5.8S gene region, the internal transcribed spacer ITS1 and ITS2). Genomic DNA Miniprep kit (Bio Basic Canada Inc., Markham, Ontario, Canada). The gene regions were amplified with primers ITS 1f and ITS4 for ITS and LROR and LR7 for nLSU.

2.3. Fermentation

Sanghuangporus sp. was cultivated in 500 mL Erlenmeyer flask containing 200 mL of the three different liquid media YMG, Q6 ½ and ZM ½ (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 each flask. 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 five days after glucose depletion.

2.4. Extraction of crude extracts from small scale fermentation

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 compound on the ZM ½ supernatant crude extract (Dictionary of Natural Products on DVD, 2017).

2.5. Scale-up 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 500 mL Erlenmeyer flask containing 200 mL (30 flasks) of ZM ½ medium. The culture was 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 five days after glucose depletion.

2.6. Isolation of compounds 1–7

The supernatant culture crude extracts (700 mg) were fractionated using preparative reverse phase liquid chromatography (PLC 2020, Gilson, Middleton, USA). VP Nucleodur 100-5C 18 ec column (250 × 40 mm, 7 µm: Macherey-Nagel) used. 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 52 min and thereafter isocratic condition at 100% solvent B for 10 min. UV detection was carried out at 210, 254 and 350 nm and flow rate 35 mL/min. Five fractions (F1-F5) were collected according to the observed peaks.

Fraction F1 and F2 were further purified by reversed phase LC (solvent A/solvent B), elution gradient 20–30% solvent B for 30 min followed by gradient shift from 35 to 100% in 3 min and finally isocratic condition at 100% solvent B for 5 min with a preparative Nucleodur Phenyl hexyl column (Macherey-Nagel, Düren, Germany; 250 × 21 mm, 5 µm) as stationary phase and a flow rate of 15 mL/min, to afford compound **1** (3 mg) and compound **3** (50 mg). Using the same column and a modified elution gradient (25–45% solvent B for 30 min fraction) F3 was purified to afford 45 mg of **2**. Fractions F4 and F5 were purified by reversed phase HPLC (solvent A/solvent B), elution gradient 78–100% solvent B for 25 min followed by isocratic condition at 100% solvent B for 5 min with a preparative (Kromasil, MZ Analysentechnik, Mainz, Germany) 250 × 20 mm 7 µm C-18 column as stationary phase to give compound **4** (125 mg) and **5** (10 mg). The same separation and purification conditions were applied to the mycelial culture. Compound **6** (5 mg) and **7** (2 mg) were purified from F4 by reverse phase LC (solvent A/solvent B), elution gradient 65–85% solvent B for 20 min followed by gradient shift from 85 to 100% in 3 min and finally isocratic condition at 100% solvent B for 5 min with a preparative HPLC column (Kromasil, 250 × 20 mm, 7 µm C-18) as stationary phase.

2.7. Antimicrobial assay

Minimum Inhibition Concentrations (MIC) against different test organisms were determined in serial dilution assay as described previously by Teponno et al. (2017), against *Candida tenuis* MUCL 29982, *Mucor plumbeus* MUCL 49355, *Escherichia coli* DSM498 and *Bacillus subtilis* DSM10 and *Micrococcus luteus* DSM 1790. The assays were carried out in 96-well microtiter plates in YMG medium for filamentous fungi and yeasts and MH for bacteria. The stock solution concentration was 300 µg/mL.

2.8. Nematicidal assay

Compounds **1-5** were assessed for nematicidal activity against *Caenorhabditis elegans* according to Rupcic et al. (2018) and Kuephadungphan et al. (2017) with slight modification. *Caenorhabditis elegans* were inoculated monoxenically on nematode agar at room temperature for 4–5 days. Thereafter, nematodes were washed down from the plates with M9 buffer. The final nematodes concentration was adjusted to 500 nematodes/mL of M9 buffer. Assay was performed in