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Hypoxyvermelhotins A–C, new pigments from *Hypoxylon lechatii* sp. nov

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ABSTRACT

A new species of *Hypoxylon* was discovered, based on material collected in French Guiana and recognised on the basis of new combination of morpholological characters in comparison with type and authentic material of macroscopically similar taxa. These findings were corroborated by the rather isolated positions of its ITS-nrDNA and beta-tubulin DNA sequences in molecular phylogenies. However, the most salient feature of this fungus only became evident by a comparison of its stromatal HPLC profile, revealing several secondary metabolites that were hitherto not observed in stromata of any other member of the *Xylariaceae*. Part of the stromata were subsequently extracted to isolate these apparently specific components, using preparative chromatography. Five metabolites were obtained in pure state, and their chemical structures were elucidated by means of high resolution mass spectrometry and nuclear magnetic resonance spectroscopy. They turned out to be tetramic acid derivatives of the so-called vermelhotin type. Aside from vermelhotin, previously isolated from cultures of endophytic fungi, we identified three novel congeners, for which the trivial names hypoxyvermelhotins A–C were proposed. Like vermelhotin, they constitute orange-red pigments and a preliminary biological characterisation revealed them to have rather strong cytotoxic and moderate to weak antimicrobial effects. These results further illustrate the high diversity of unique secondary metabolites in stromata of the hypoxyloid *Xylariaceae*, a family in which biological diversity seems to parallel the chemical diversity of their bioactive principles to a great extent.

Introduction

With more than 1300 accepted species accommodated in over 70 genera, the family *Xylariaceae* is one of the largest within the Ascomycota (for their current arrangement in view of the recent nomenclatural changes following the IBC at Melbourne 2011, see Stadler *et al.* 2013). The mainstream of the family is divided into two major groups, i.e., the informal subfamilies *Xylarioideae* and *Hypoxyloideae*, which are represented by two extremely large genera: *Xylaria* (geniculosporium-like anamorph types) and *Hypoxylon* (nodulisporium-like anamorph types), and their respective allies. While no world monograph of *Xylaria* is available, the current generic concept of *Hypoxylon* is mainly based on the monograph by Ju & Rogers (1996), except for some amendments by Hsieh *et al.* (2005), who also segregated the former section *Annulata*

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sensu Ju & Rogers 1996 from *Hypoxylon* as inferred from a molecular phylogeny based on β-tubulin and α-actin gene sequences. This resulted in the erection of *Annulohypoxylon*. The *Hypoxyloideae*, and in particular the genus *Hypoxylon* itself, are characterised by a particular high diversity of secondary metabolites, and overproduce manifold unique pigments and other biologically active compounds in their stromata (Stadler & Hellwig 2005; Stadler & Fournier 2006; Stadler 2011).

Ju & Rogers (1996) introduced colours of KOH-extractable stromatal pigments as chemotaxonomic discriminative characters for *Hypoxylon* taxa. These pigments are located on the stromatal surface and/or in subsurface granules surrounding the perithecia. However, a certain colour in KOH may result from various different chemical entities, hence secondary metabolite profiling based on high performance liquid chromatography coupled to diode array and mass spectrometric detection (HPLC–DAD/MS) has provided a much higher resolution and could frequently help to resolve species complexes in *Hypoxylon* and other hypoxyloid *Xylariaceae* (cf. Stadler *et al.* 2004, 2008; Fournier *et al.* 2010; Stadler *et al.* 2014).

The HPLC–DAD/MS technique is not more invasive than microscopy, allowing for detection of secondary metabolites in trace amounts.

In the course of chemotaxonomic studies it was often possible to detect apparently specific, yet unknown metabolites that most likely constitute novel natural products. This was already demonstrated by preparative mycochemical work on various species, yielding numerous unprecedented bioactive molecules. However, most of the work hitherto conducted on the preparative isolation of specific metabolites from stromata of *Hypoxylon* and its allies were carried out on species whose stromata commonly occur in the Northern temperate hemisphere. Their tropical relatives remained widely untapped. An exception is *H. carneum*, which is a rare species of apparently cosmopolitan occurrence (Quang *et al.* 2006), but its stromata were found in relatively large quantities in France. This allowed for identification of its specific metabolites (carneic acids A and B) after extensive preparative chromatography of the stromatal extracts and subsequent structure elucidation of the purified metabolites by spectral methods. On the other hand, the identification of the major stromatal metabolites of *H. aeruginosum* and the similar species of *Chlorostroma* as lepraric acids and their derivatives was accomplished due to the availability of standards that were originally isolated from lichens (Læssøe *et al.* 2010). The same was accomplished by Fournier *et al.* (2010), who found lecanoric acids, which are widely distributed in lichenised ascomycetes, for the first time as apparently species-specific major stromatal metabolites of *H. addis*.

The current study deals with a species group in the *H. rubiginosum* complex *sensu* Ju & Rogers (1996) that is predominantly distributed in warmer climates: *Hypoxylon anthochroum*, *H. duranii* and morphologically similar taxa. Stadler *et al.* (2008) already evaluated several collections of *H. anthochroum* and found inconsistent HPLC profiles in a range of specimens from different geographic regions, some of which contained apparently specific metabolites. This prompted us to study fresh material superficially resembling *H. anthochroum* carefully in attempt to find hitherto undescribed taxa that are capable of producing unprecedented biologically active secondary metabolites. The current study on material from French Guiana deals with the characterisation of such a fungus and its major stromatal pigments.

Materials and methods

General

If not indicated otherwise solvents were obtained in analytical grade from J.T.Baker (Deventer, Netherlands) or Merck (Darmstadt, Germany). Bold numbering refers to the chemical structures depicted in Fig 1. All scientific names of fungi follow the entries in Mycobank (*www.***mycobank***.org*), hence no authorities and years of publications are given. Reference specimens including the designated type material of the new species, are housed in LIP (University of Lille, France) and corresponding reference cultures have been deposited with CBS (Utrecht, The Netherlands) or MUCL (Louvain, Belgium); see Kuhnert *et al.* (2014) for further details. Acronyms of herbaria and culture collections are given as recommended in Index Herbariorum (http://sciweb.nybg.org/science2/IndexHerbariorum.asp).

Morphological characterisation

Fungal material was examined for macro- and micromorphological features as described by Fournier *et al.* (2010). Photos of asci and ascospores were taken through a brightfield microscope at 400–1000× magnification. Cultures were grown from multispore isolates incubated on YMG agar plates (Bitzer *et al.* 2008) and also transferred to Difco Oatmeal agar (OA) in 9 cm petri dishes for observation of the macroscopic morphology using a stereomicroscope and for anamorphic characters using differential interference contrast under 400–1000× optical magnification. Pigment colours were determined as in the monograph by Ju & Rogers (1996) and, accordingly, colour codes follow Rayner (1970).

HPLC profiling

Parts of the stromata were extracted with methanol as described by Stadler *et al.* (2001) and analysed using an analytical HPLC instrument (Agilent 1260 Infinity Series) equipped with a diode array detector and an ESI-iontrap MS detector (Amazon, Bruker). The instrumental settings were the same as described for the high resolution Electrospray ionisation mass spectrometry (HR-ESIMS) by Pažoutová *et al.* (2013). Spectra were compared to an internal database comprising standards of known secondary metabolites produced by *Xylariaceae* from previous work (Bitzer *et al.* 2007).

Molecular phylogenetic analyses

DNA was isolated from pure cultures grown on YMG. Small amounts of mycelia were transferred to a 1.5 ml homogenisation tube filled with eight Precellys Ceramic beads (1.4 mm, Bertin Technologies, Montigny-le-Bretonneux, France). DNA extraction was performed using the Invisorb Spin Plant Mini Kit (STRATEC, Birkenfeld, Germany) according to the manufacturers' recommendations, but with the following