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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 the Caribbean and French Guiana and recognised on the basis of new combination of morphological 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 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.* 2013a). The mainstream of the family is divided into two major groups, i.e., the informal subfamilies *Xylaroideae* 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 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 entites, 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.* 2008, Fournier *et al.* 2010, Stadler *et al.* 2013b).

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 lepralic 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 and Martinique 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.* (2013) 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 8 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 modifications: no proteinase K and only 50 µl of the elution buffer were used. The Precellys 24 Homogenizer (Bertin Technologies) was used for cell disruption at a speed of 6000 rpm for 2 × 40 s. The ITS region (ITS1, 5.8S and ITS2 rRNA gene) and the β-tubulin gene of the extracted DNA was amplified by using the following primer combinations: ITS4 (White *et al.* 1990) and ITS1F (Gardes & Bruns 1993), T1 and T22 (O'Donnell & Cigelnik 1997), respectively. The reaction mix for PCR (total volume of 50 µl) contained 2 µl template DNA, 21 µl water (nuclease-free, sterile filtered and steam sterilised, DEPC treated; Roth, Karlsruhe, Germany), 25 µl JumpStart Taq ReadyMix (Sigma-Aldrich, Deisenhofen, Germany), 1 µl forward primer (10 µM) and 1 µl reverse primer (10 µM). Amplification was performed with a Mastercycler nexus Gradient (Eppendorf, Hamburg, Germany).

Thermocycling for ITS commenced with an initial denaturation at 95 °C for 5 min followed by 34 cycles of denaturation (30 s at 94 °C), annealing (30 s at 52 °C) and elongation (2 min at 72 °C). The last step was a 10 min lasting elongation at 72 °C. Afterwards reaction tubes were cooled to 10 °C. In case of the β -tubulin amplification cycles were raised to 36, annealing temperature was set to 47° and elongation was extended to 2.5 min. PCR products were checked using gel electrophoresis with 0.8% agarose gels (Life Technologies, Darmstadt, Germany) in 0.5% TAE buffer (TRIS-acetate-EDTA buffer) for 45 min at 80 V. Bands were visualized with SYBR Green I (Roche Applied Science, Penzberg, Germany) according to the manufacturer's protocol under UV light. Amplification products were purified using Nucleo Spin Gel and PCR Clean Up (Macherey-Nagel, Düren, Germany) as specified in the manufacturers' recommendations. 39 sequences of each gene originating from reliable sources (Bills *et al.* 2012, Bitzer *et al.* 2008, Hsieh *et al.* 2005, Kuhnert *et al.* 2013) were used in addition for phylogenetic analysis. Details on the considered specimens are given in Table 1. Calculations were performed as described in Kuhnert *et al.* (2013) by using maximum likelihood (ML) as optimality criterion. Phylogenetic reconstruction of the ML tree was achieved with the software RAxML (Stamatakis 2006) applying the GTRCAT rate distribution model (with 25 rate categories) and the rapid bootstrap analysis algorithm. The program executed 1,000 bootstrap replicates, the support values of which were mapped onto the most likely tree topology found by an independently executed search. Branch lengths were thereby calculated based on base exchange rates.

Extraction and isolation of compounds 1-5: A large stroma (approx. 6 × 3 cm) of specimen CLL8035 was detached from the woody substrate and extracted with 200 ml methanol in an ultrasonic bath for 30 min at 40 °C. The fruiting bodies were filtered out and the previous procedure was repeated two times in addition. The resulting oily crude extracts (ca. 120 mg) were combined, filtered and evaporated *in vacuo* to yield 400 mg of crude product. The latter

was filtered again through a RP solid phase cartridge (Strata-X 33 μ m, Polymeric Reversed Phase; Phenomenex Aschaffenburg, Germany) and subjected to preparative HPLC (PLC 2020, Gilson, Middleton, USA). A VP Nucleodur C18 ec column (125 x 40 mm, 7 μ m; Macherey-Nagel) was used as stationary phase. The mobile phase was composed of deionised water (Milli-Q, Millipore, Schwalbach, Germany) with 0.1% acetic acid (solvent A; Roth) and acetonitrile with 0.1% acetic acid (solvent B). A flow rate of 30 ml/min was used for the following gradient: 10% to 60% solvent B in 25 min, afterwards linear gradient to 100% solvent B in 5 min, thereafter isocratic conditions at 100% for 10 min. UV detection was carried out at 210 nm and 254 nm and fractions were collected and combined according to the observed peaks. The separation yielded five fractions with (**1**; 19.5 mg) at a retention times (R_t) = 16–18 min, (**2**; 1.4 mg) at R_t = 10–11 min, (**3**; 1.0 mg) at R_t = 13 min, (**4**; 1.4 mg) at R_t = 14–15 min and (**5**; 2.5 mg) at R_t = 28 min.

Hypoxyvermelhotin A (**1**)

(*E:Z* mixture); orange amorphous solid ; UV/Vis (MeOH) $\lambda_{\max}$ (log ϵ) 279 (3.47), 321 (2.56), 444 (3.45); IR $\nu_{\max}$ 3176, 3046, 2927, 2852, 1690, 1641, 1607, 1557, 1491, 1445, 1385, 1253, 1209, 965, 846, 767, 713, 658 cm^{-1} ; *E*-isomer: ^1H , ^{13}C NMR see Table 1; *Z*-isomer: ^1H -NMR (CHCl_3 -*d*; 700 MHz) δ 8.18 (1H, d, J = 9.5, 7-H), 7.47 (1H, dq, J = 15.3, 7.1, 12-H), 7.33 (1H, d, J = 9.5, 8-H), 6.37 (1H, dq, J = 15.3, 1.5, 11-H), 5.29 (1H, brs, NH), 3.81 (2H, d, J = 0.9, 5- H_2), 2.17 (3H, s, 9-Me), 2.04 (3H, dd, J = 7.1, 1.5, 13- H_3); ^{13}C -NMR (CHCl_3 -*d*; 175 MHz) δ 194.6 (qC, C-4), 171.7 (qC, C-2), 166.1 (qC, C-6), 155.7 (qC, C-10), 146.1 (CH, C-8), 139.6 (CH, C-12), 119.3 (CH, C-11), 116.5 (qC, C-9), 115.7 (CH, C-7), 97.3 (qC, C-3), 50.9 (CH_2 , C-5), 19.5 (CH_3 , C-13), 15.6 (CH_3 , 9-Me); ESI-MS m/z 232.09 $[\text{M}+\text{H}]^+$; 229.99 $[\text{M}-\text{H}]^-$; HR-ESI-MS m/z 232.0968 $[\text{M}+\text{H}]^+$ (calcd. for $\text{C}_{13}\text{H}_{14}\text{NO}_3$, 232.0968).

Hypoxyvermelhotin B (**2**)

(*E:Z* mixture); yellow amorphous solid; $[\alpha]_D^{25} +0.1$ (*c* 0.1, CH₃OH); UV/Vis (MeOH) $\lambda_{\max}$ (log ϵ) 240 (3.58), 284 (3.28), 297 (3.30), 423 (3.76); IR $\nu_{\max}$ 3189, 3042, 2925, 1688, 1642, 1557, 1488, 1250, 1201, 1045, 981, 847, 769, 716, 658 cm⁻¹; *E*-isomer: ¹H, ¹³C NMR see Table 1; *Z*-isomer: ¹H-NMR (CHCl₃-*d*; 700 MHz) δ 8.21 (1H, d, *J* = 9.5, 7-H), 7.39 (1H, d, *J* = 9.5, 8-H), 5.65 (1H, brs, NH), 4.41 (1H, m, 12-H), 3.80 (2H, brs, 5-H₂), 2.91 (2H, m, 11-H₂), 2.11 (3H, s, 9-Me), 1.34 (3H, d, *J* = 6.2, 13-H₃); ¹³C-NMR (CHCl₃-*d*; 175 MHz) δ 194.0 (qC, C-4), 172.2 (qC, C-2), 166.2 (qC, C-6), 161.6 (qC, C-10), 145.8 (CH, C-8), 117.8 (qC, C-9), 116.2 (CH, C-7), 96.9 (qC, C-3), 63.8 (CH, C-12), 50.9 (CH₂, C-5), 40.2 (CH₂, C-11), 23.4 (CH₃, C-13), 15.8 (CH₃, 9-Me); ESI-MS *m/z* 250.09 [M+H]⁺; 248.02 [M-H]⁻; HR-ESI-MS *m/z* 250.1074 [M+H]⁺ (calcd. for C₁₃H₁₆NO₄, 250.1074).

Hypoxyvermelhotin C (**3**)

(*E:Z* mixture); yellow amorphous solid ; $[\alpha]_D^{25} +0.5$ (*c* 0.1, CH₃OH); UV/Vis (MeOH) $\lambda_{\max}$ (log ϵ) 240 (3.39), 298 (3.11), 422 (3.57); IR $\nu_{\max}$ 3190, 3045, 2925, 1692, 1639, 1556, 1499, 1459, 1385, 1253, 1204, 1133, 1096, 972, 847, 770, 709, 657 cm⁻¹; *E*-isomer: ¹H, ¹³C NMR see Table 1; *Z*-isomer: ¹H-NMR (CHCl₃-*d*; 700 MHz) δ 8.25 (1H, d, *J* = 9.5, 7-H), 7.33 (1H, d, *J* = 9.5, 8-H), 5.31 (1H, brs, NH), 4.16 (1H, m, 12-H), 3.80 (2H, brs, 5-H₂), 3.34 (3H, brs, 12-OMe), 2.97 (1H, m, 11-H_a), 2.78 (1H, m, 11-H_b), 2.15 (3H, brs, 9-Me), 1.27 (3H, d, *J* = 6.0, 13-H₃); ¹³C-NMR (CHCl₃-*d*; 175 MHz) δ 194.6 (qC, C-4), 171.4 (qC, C-2), 167.2 (qC, C-6), 160.2 (qC, C-10), 145.5 (CH, C-8), 118.3 (qC, C-9), 116.6 (CH, C-7), 97.2 (qC, C-3), 74.4 (CH, C-12), 56.6 (CH₃, 12-OMe), 50.9 (CH₂, C-5), 38.3 (CH₂, C-11), 19.3 (CH₃, C-13), 16.1 (CH₃, 9-Me); ESI-MS *m/z* 264.12 [M+H]⁺; 262.04 [M-H]⁻; HR-ESI-MS *m/z* 264.1232 [M+H]⁺ (calcd. for C₁₄H₁₈NO₄, 264.1230).

Vermelhotin (**4**)

^1H NMR and ^{13}C NMR data were consistent with those previously reported (Hosoe *et al.* 2006; Kasettrathat *et al.* 2008); ESI-MS m/z 218.08 $[\text{M}+\text{H}]^+$; 215.99 $[\text{M}-\text{H}]^-$; HR-ESI-MS m/z 218.0811 $[\text{M}+\text{H}]^+$ (calcd. for $\text{C}_{12}\text{H}_{12}\text{NO}_3$, 218.0812).

Structure elucidation: Optical rotations were determined with a Perkin-Elmer 241 polarimeter, IR spectra were measured with a Spectrum 100 FTIR spectrometer manufactured by Perkin Elmer (Waltham, MA, USA), UV-Vis spectra were recorded with a Shimadzu (Kyoto, Japan) UV-Vis spectrophotometer UV-2450. NMR spectra were recorded with a Bruker (Bremen, Germany) Ascend 700 spectrometer with a 5 mm TXI cryoprobe (^1H 700 MHz, ^{13}C 175 MHz). ESIMS spectra were obtained with an ion trap MS (Amazon, Bruker), HRESIMS spectra were obtained with a time-of-flight MS (Maxis, Bruker) as described by Pažoutová *et al.* (2013).

RESULTS

Structure elucidation: The main metabolite hypoxyvermelhotin A (**1**) exhibited maxima at 226, 280 and 456 nm in the UV-Vis spectrum and yielded ESIMS signals at m/z 232.09 $[\text{M}+\text{H}]^+$ and 229.99 $[\text{M}-\text{H}]^-$. Its molecular formula was found to be $\text{C}_{13}\text{H}_{13}\text{NO}_3$ on the basis of its $[\text{M}+\text{H}]^+$ HRESIMS signal at m/z 232.0968, thereby indicating eight degrees of unsaturation. The ^1H NMR (CDCl_3) showed spontaneous interconversion between *E* and *Z* isomers. Because the isomers were not separable by HPLC, the structural analysis was carried out on the equilibrium *E/Z* mixture. The two sets of resonances in the ^1H and ^{13}C spectra revealed a ratio of 1/0.6 for *E* and *Z* isomers. The structure elucidation of **1** is described as

follows with data of the more abundant *E* isomer. The ^1H NMR (Tab. 1) spectrum identified four olefinic signals [δ_{H} 8.17 (d, $J=9.5$ Hz, 1H), 7.52 (dq, $J=15.3$, 7.1 Hz, 1H), 7.33 (d, $J=9.5$ Hz, 1H), 6.39 (dq, $J=15.3$, 1.5 Hz, 1H)], a sp^3 methylene [δ_{H} 3.79 (d, $J=0.9$ Hz, 2H)], two methyls [δ_{H} 2.17 (s, 3H), 2.06 (dd, $J=7.1$, 1.5 Hz, 3H)] and one amide proton [δ_{H} 5.39 (brs, 1H)]. The ^{13}C and DEPT (Distortionless Enhancement by Polarization Transfer) spectra established the presence of an unsaturated ketone (δ_{C} 192.5), an amide/ester carbonyl (δ_{C} 172.8), four quaternary sp^2 carbons (δ_{C} 165.0, 155.6, 116.5, 97.5), two of which were oxygenated, four sp^2 methines (δ_{C} 145.6, 138.8, 119.3, 116.0), one sp^3 methylene (δ_{C} 50.4) and two methyl groups (δ_{C} 19.4, 15.6). The COSY (Correlation Spectroscopy) spectrum showed the connections of NH/5-H, 7-H/8-H and 12-H/13-H (Figure 2). The tetramic acid moiety was established by HMBC correlations from 5-H to C-2 and C-4 respectively from NH to C-3, C-4 and C-5. The partial structure of the 7-methyl substituted pyrone ring with C-11 to C-13 side chain was recognised by HMBC (Heteronuclear Multiple Bond Correlation) correlations from 7-H to C-6 and C-9 respectively from 9-Me to C-8, C-9 and C-10; the ring closure resulted from one missing unit of unsaturation. Despite no HMBC correlation was observed from 7-H to quaternary C-3, the information of the presence of an unsaturated ketone as well as the open valences required a connection of C-3 and C-6. Therefore, the structure of **1** was elucidated as 3-(5-methyl-6-[(1*E*)-prop-1-en-1-yl]-2*H*-pyran-2-ylidene)pyrrolidine-2,4-dione.

The HRESIMS of hypoxyvermelhotin B (**2**) showed a $[\text{M}+\text{H}]^+$ peak at m/z 250.1074, which indicated a molecular formula of $\text{C}_{13}\text{H}_{15}\text{NO}_4$ and implied a formal addition of H_2O compared to **1**. The ^1H and ^{13}C NMR data (Tab. 1) of **2** resemble those of **1**, with the exception of the replacement of olefinic signals C-11 and C-12 with sp^3 methylene [δ_{C} 40.2; δ_{H} 2.91 (m, 2H)] and oxygenated sp^3 methine [δ_{C} 63.6; δ_{H} 4.41 (m, 1H)] signals. The HMBC correlation from

13-H to C-11 and C-12 together with the COSY correlation from 13-H to 12-H established the structure as 3-[6-(2-hydroxypropyl)-5-methyl-2*H*-pyran-2-ylidene]pyrrolidine-2,4-dione. The molecular formula C₁₄H₁₇NO₄ of hypoxyvermelhotin C (**3**) was determined from the HRESIMS [M+H]⁺ peak at *m/z* 264.1230. The NMR data (Tab. 1) for this compound were very nearly identical with those of **2**, with the exception of an additional oxymethyl group [δ_c 56.6; δ_H 3.34 (s, 3H)]. The spectroscopic differences are due to the substitution of the hydroxyl group of C-12 by a methoxy group, which was proven by HMBC correlations from 12-OMe to C-12, ascertaining the structure of **3** as 3-[6-(2-methoxypropyl)-5-methyl-2*H*-pyran-2-ylidene]pyrrolidine-2,4-dione.

Metabolite **4** was identified as vermelhotin on the basis of its UV, NMR and MS data by comparison with the literature (Hosoe *et al.* 2006; Kasettrathat *et al.* 2008). **5** was identified as BNT through a comparative search with standard compounds derived from Xylariaceae available at our department.

Biological Activity: Pigments **1** – **4** were screened for cytotoxicity against the murine fibroblast cell line L-929 as described by Okanya *et al.* (2011). Only **1** and **4** showed moderate cytotoxicity (**1**: 5 μ g/ml, **4**: 2 μ g/ml). Both metabolites were furthermore evaluated for their antibiotic activity against Gram-positive and Gram-negative bacteria, yeasts and filamentous fungi with our standard panel of test organisms (Surup *et al.* 2013).

Hypoxyvermelhotin A (**1**) exhibited a weak activity against *Mucor hiemalis* DSM 2656 (67 μ g/mL) and *Nematospora coryli* DSM 6981 (33.3 μ g/mL). Vermelhotin (**4**) showed weak activity against *Mucor hiemalis* DSM 2656 (33.3 μ g/mL).

Hypoxydon lechatii J. Fourn. & M. Stadler, **sp. nov.**

MycoBank MB80558

Figs. 2 & 3

Etymology: In honour of our colleague, the French mycologist Christian Lechat, who collected this fungus for the first time.

A Hypoxydon anthochroum et H. macrocarpum differt ostiolis papillatis et stromata ad 2mm crassa. Granulis hypoxyvermelhotinis praesunt.

Holotype: French Guiana: Sinnamary: Guyaflux plots, on bark of unknown host, associated with old stromata of *Camillea heterostoma*, 30 Aug. 2008, leg C. Lechat, in herb J. Fournier CLL8035 (LIP, ex-type culture CBS 123577; GenBank Acc. No: XXX¹).

Known distribution/host preference of stromata: French Guiana, host unknown.

Teleomorph: Stromata widely effused-pulvinate with lobate margins, 15–100 mm long, 1.6–2 mm thick, surface pruinose, Brown Vinaceous (84), slightly nodulose with bluntly protruding ostioles; dull orange brown granules beneath the surface and between perithecia yielding Olivaceous (48) pigments in 10% KOH; the subperithecial tissue blackish, woody, 0.3–0.6 mm thick. *Perithecia* long tubular, 1.3–1.6 mm high x 0.3–0.4 mm diam. *Ostioles* bluntly papillate, opening higher than stromatal surface, forming discoid areas 160–200 µm diam on stromatal surface. *Asci* unitunicate, 8-spored, the spore-bearing parts 57–63 µm long x 5–6 µm broad, the stipes 50–110 µm long very fragile, originating from long ascogenous hyphae, with apical ring discoid, 0.8–1 µm high, 1.7–2 µm wide, bluing in Melzer's reagent.

Ascospores 8.5–10 x 4–4.5 µm, ellipsoid-inequilateral with narrowly rounded ends, often slightly twisted, dark brown, with a faint straight germ slit spore-length; perispore dehiscent in 10% KOH, smooth.

¹ The sequences were submitted but no acc nos were obtained from GenBank yet. They will be inserted as they become available, hopefully until the galley proofs arrive.

Cultures and anamorph: Colonies on OA covering a 9 cm Petri dish after 2 weeks, at first white, becoming Buff (46) to Grey Olivaceous (107). No conidiogenous structures were observed after 4 weeks of cultivation.

Secondary metabolites: Stromata contain hypoxyvermelhotin A (**1**) and BNT (**5**) as main compounds. Furthermore vermelhotin (**4**), hypoxyvermelhotin B (**2**) and C (**3**) can be detected in small amounts. 3-hydroxyisosclerones, 5-methylmellein and unknown metabolites were observed in liquid YMG cultures.

Additional specimens examined: **French Guiana**, Les Nouragues, forest margin, on dead wood, 17 Jun. 2012, J. Fournier GYJF12012 (LIP, culture MUCL 54609; GenBank Acc. No: ITS – XXX, β -tubulin – YYY²).

Notes: *Hypoxylon lechatii* strongly resembles *H. anthochroum* and *H. macrocarpum* in possessing brown vinaceous effused-pulvinate stromata and similar ascospores. However both differ in having thinner stromata and much smaller perithecia and lack the papillate ostioles of the present fungus. The occurrence of vermelhotin type tetramic acids is rather unique in *Hypoxylon*. The new species can also easily be segregated from *H. macrocarpum* by the lack of macrocarpones (**8**), as well as from all chemotypes of *H. anthochroum sensu* Ju & Rogers (1996), by the lack of daldinins (**6**, **7**). The position of the new species is unstable in the ITS phylogeny. Especially the β -tubulin DNA sequence shows higher homologies to those of *Daldinia* spp. than to other *Hypoxylon* spp. However, even this relationship is neither corroborated by morphological nor by chemical features. A recent study by Kuhnert *et al.* (2013) has suggested that β -tubulin data are better suited to reflect the taxonomy and phylogeny of *Hypoxylon* than ITS sequences. However, the current species concepts in *Xylariaceae* rely exclusively on phenotype-derived data, also because no DNA sequences of the majority of species are available as yet (cf. Stadler *et al.* 2013).

² The sequences were submitted but no acc nos were obtained from GenBank yet. They will be inserted as they become available

Molecular phylogeny: In total, DNA sequences derived from 40 strains of hypoxyloid *Xylariaceae* (Stadler *et al.* 2013a) were selected for each gene (ITS and β -tubulin, respectively). For the ITS data set one additional strain of *H. lechatii* (CBS 123577) was sequenced. Those comprised the genera *Hypoxylon* (29 taxa), *Annulohypoxylon* and *Daldinia* (4 taxa each), and one sequence of a representative of the xylarioid genus *Nemania* which was used for rooting.

Our taxon selection was restricted to neotropical species, except for the type species of the four genera and *Daldinia childiae*, which is known to have a wide distribution in warmer climates (Stadler *et al.* 2013b). In both trees (Figs. 4 & 5) the backbone is not well-supported by statistical means, but the overall bootstrap (BS) support for the subclades is higher in the β -tubulin (TUB) reconstruction. The ITS tree differs in many positions from the TUB tree. However in both calculations the *Daldinia* spp. form a monophyletic clade and *Annulohypoxylon* and *Hypoxylon* appear paraphyletic. The two strains of *H. lechatii* form an independent branch in the ITS tree, which lacks BS support to adjacent clades. In the TUB reconstruction the new species is placed basal to the *Daldinia* clade with statistical support of 81%.

DISCUSSION

The new metabolites hypoxyvermelhotins A – C (**1** – **3**) represent derivatives of the tetramic acid antibiotic vermelhotin (**4**), which has been reported from three fungal endophytes (Hosoe *et al.* 2006, Kasettrathat *et al.* 2008, Leyte-Lugo *et al.* 2012). Two of those belong to the order *Pleosporales*. Representatives of this group are far related from the Xylariales, which might indicate that the biosynthetic pathway of vermelhotin (**4**) has evolved independently multiple times during the evolution of fungi. The cytotoxicity of **4** was already reported by Kasettrathat *et al.* (2008), who in addition found activity against various cancer cell lines. The

structurally very similar hypoxyvermelhotin A (**1**) showed a slightly weaker cytotoxic activity. Hypoxyvermelhotin B (**2**) and C (**3**) were virtually inactive in the cytotoxicity assay, which could be explained by a decreased permeation into lipophilic cell membranes caused by the increased polarity of the compounds. Contrariwise, the C-11/C-12 double bond could be an indispensable part of the pharmacophore. Furthermore, Leyte-Lugo *et al.* (2012) screened various fungal extracts for calmodulin inhibiting activities. In this process vermelhotin (**4**) was revealed as a calmodulin inhibitor. They suggest that the cytotoxic effect of this compound is related to its binding properties to calmodulin due to its importance for a variety of cellular functions. The structural similarity of hypoxyvermelhotins A–C (**1–3**) to vermelhotin (**4**) suggests a common biosynthesis. Tetramic acids are thought to be biosynthesised by polyketide synthase-nonribosomal peptide synthetase (PKS-NRPS) hybrid systems (Boettger & Hertweck 2013). The amino acid glycine is condensed with an unsaturated β -oxodecanoic acid, followed by the further cyclisation between 6-OH and C-10. Chain branches like methyl group 9-Me are introduced by *S*-adenosylmethionine (SAM)-derived from a methylation of the growing polyketide chain by methyltransferases. Therefore, the function of a single methyltransferase would account for the structural difference between compounds **1** and **4**. Hypoxyvermelhotins B (**2**) and C (**3**) might constitute natural products with an incomplete dehydration at the first elongation step of the biosynthesis of the growing β -oxodecanoic acid. However, the virtual absence of optical rotation for **2** and **3** makes a non-enzymatic derivation from **1** more probable.

Hypoxyvermelhotins and vermelhotin are the first tetramic acids so far known from the *Xylariaceae*. It is important to note that the cultures of *H. lechatii* were apparently devoid of these compounds. Not even the characteristic intensely orange colour was observed. In the stromata of the new species, however, the tetramic acids **1–4** appear to be highly specific chemotaxonomic markers. Similarity searches for the characteristic UV/VIS chromophore only

revealed high matches to the yet unidentified major metabolites that were detected in *H. porphyreum* besides BNT (5). However, those differ strongly in their retention times. *Hypoxylon* species and related taxa have been classified into so-called chemotypes (Hellwig *et al.* 2005, Stadler & Fournier 2006). For instance, there are entire conglomerates of species in the *H. rubiginosum* complex that have similar HPLC profiles and contain certain azaphilones such as mitorubrans, rubiginosins, and daldinins, all of which are widely distributed (see Stadler *et al.* 2008 for an overview on the temperate/subtropical species). The new species recognised in this study represents one of a few species (groups) that deviate from the mainstream of *Hypoxylon* taxa in this respect, even though the binaphthalene BNT (5), which is widely distributed in the hypoxylid Xylariaceae, was found in its stromata. However it is important to mention, that approximately only 5% of the HPLC-MS detectable compounds in *Hypoxylon* are known so far. The identification of these compounds depends on the quantity of available stromata material and the producing capacities of the corresponding organism to get sufficient amounts of pure compounds. In most cases the amount of fruiting bodies is limited in nature. In order to overcome this problem it might be reasonable to increase efforts in growing the stromata under artificial conditions.

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Tab. 1: ^{13}C NMR (ppm, mult.) and ^1H NMR (ppm, mult.) data^a for **1** – **3** (J in Hz) in CDCl_3 .

	1		2		3	
	δ_{C}	δ_{H}	δ_{C}	δ_{H}	δ_{C}	δ_{H}
2	172.8, qC		172.5, qC		172.8, qC	
3	97.5, qC		96.9, qC		97.2, qC	
4	192.5, qC		193.4, qC		192.4, qC	
5	50.4, CH ₂	3.79, d (0.9)	50.3, CH ₂	3.82, d (0.6)	50.4, CH ₂	3.76, brs
6	165.0, qC		166.9, qC		166.1, qC	
7	116.0, CH	8.17, d (9.5)	115.8, CH	8.21, d (9.5)	116.1, CH	8.21, d (9.5)
8	145.7, CH	7.33, d (9.5)	145.7, CH	7.37, d (9.5)	145.1, CH	7.31, d (9.5)
9	116.5, qC		117.6, qC		118.0, qC	
10	155.6, qC		161.5, qC		160.0, qC	
11	119.3, CH	6.39, dq (15.3, 1.5)	40.2, CH ₂	2.91, m	38.2, CH ₂	2.97, m
12	138.8, CH	7.52, dq (15.3, 7.1)	63.6, CH	4.41, m	74.3, CH	2.78, m
13	19.4, CH ₃	2.06, dd (7.1, 1.5)	23.4, CH ₃	1.36, d (6.2)	19.4, CH ₃	4.16, m
9-Me	15.6, CH ₃	2.17, s	15.7, CH ₃	2.10, s	16.1, CH ₃	1.28, d (6.0)
12-OMe					56.6, CH ₃	2.14, brs
NH		7.38, brs		5.64, brs		3.34, s
						5.44, brs

^a Assignments confirmed by 2D COSY, HSQC, HMBC experiments.

Tab. 2: List of used taxa for the phylogenetic reconstruction. GenBank accession numbers, strain number of public culture collections, origin and reference studies are given. Type specimens are labelled with **T** (holotype), **IT** (isotype) and **ET** (epitype). Scientific names follow Index Fungorum and Mycobank.

Species	GenBank Acc.. Nos.		Strain no	Origin	Reference
	β -tubulin	ITS			
<i>Annulohypoxyton annulatum</i>	KC977276	AM749938	MUCL 47218	China	Bitzer <i>et al.</i> (2008), Kuhnert <i>et al.</i> (2013)
<i>A. nitens</i>	KC977275	KC968927	CBS 119134	Guadeloupe	Kuhnert <i>et al.</i> (2013)
<i>A. purpureopigmentum</i>	KC977306	KC968942	MUCL 54616	French Guyana	Kuhnert <i>et al.</i> (2013)
<i>A. stygium</i>	KC977304	KC968940	MUCL 54600	French Guyana	Kuhnert <i>et al.</i> (2013)
<i>Daldinia andina</i> ^a	KC977259	AM749918	CBS 114736	Ecuador	Bitzer <i>et al.</i> (2008) as <i>D. grandis</i> , Kuhnert <i>et al.</i> (2013)
<i>D. childiae</i>	AY951691	-	BCRC 34044	Hawaii (USA)	Hsieh <i>et al.</i> (2005)
<i>D. concentrica</i>	-	JX658462	MUCL 46818	France	Stadler <i>et al.</i> (2013b)
	KC977274	AY616683	CBS 113277	Germany	Triebel <i>et al.</i> (2005), Kuhnert <i>et al.</i> (2013)
<i>D. placentiformis</i>	KC977278	AM749921	MUCL 47603	Mexico	Bitzer <i>et al.</i> (2008), Kuhnert <i>et al.</i> (2013)
<i>Hypoxyton aeruginosum</i>	KC977305	KC968941	MUCL 54620	French Guiana (ET)	Kuhnert <i>et al.</i> (2013)
<i>H. brevisporum</i>	AY951705	JN660821	BCRC 33809	Puerto Rico	Hsieh <i>et al.</i> (2005)
<i>H. cinnabarinum</i>	AY951709	JN979409	BCRC 33810	Mexico	Hsieh <i>et al.</i> (2005)
<i>H. dieckmanii</i>	AY951712	JN979412	BCRC 34058	Mexico	Hsieh <i>et al.</i> (2005)
<i>H. erythrostroma</i>	KC977296	KC968910	MUCL 53759	Martinique	Kuhnert <i>et al.</i> (2013)
<i>H. fendleri</i>	KF300547	KF234421	MUCL 54792	French Guiana	Kuhnert <i>et al.</i> (2013)
<i>H. fragiforme</i>	AY951719	-	BCRC 34065	France	Hsieh <i>et al.</i> (2005)
	-	AY616690	CBS 114745	Germany	Triebel <i>et al.</i> (2005)
<i>H. griseobrunneum</i>	AY951703	JN660819	BCRC 34050	Mexico	Hsieh <i>et al.</i> (2005), as <i>Hypoxyton anthochroum</i>
	KC977281	KC968928	MUCL 53310, CBS 129346	Guadeloupe	Kuhnert <i>et al.</i> (2013)
<i>H. haematostroma</i>	KC977291	KC968911	MUCL 53301	Martinique (ET)	Kuhnert <i>et al.</i> (2013)
	KC977279	AM749924	MUCL 47600	Panama	Bitzer <i>et al.</i> (2008), Kuhnert <i>et al.</i> (2013)
<i>H. hypomiltum</i>	KC977298	KC968914	MUCL 53312, CBS 129036	Guadeloupe	Kuhnert <i>et al.</i> (2013)
<i>H. investiens</i>	KC977293	KC477239	MUCL 53307, CBS 129034	Martinique	Kuhnert <i>et al.</i> (2013)
	KC977269	KC968924	CBS 118185	Ecuador	Kuhnert <i>et al.</i> (2013)
<i>H. isabellinum</i>	KC977295	KC968935	MUCL 53308, CBS 129035	Martinique (T)	Kuhnert <i>et al.</i> (2013)
<i>H. jecorinum</i>	AY951731	JN979429	N/A	Mexico	Hsieh <i>et al.</i> (2005)
<i>H. laminosum</i>	KC977292	KC968934	MUCL 53305, CBS 129032	Martinique (T)	Kuhnert <i>et al.</i> (2013)
<i>H. lateripigmentum</i>	KC977290	KC968933	MUCL 53304, CBS 129031	Martinique (T)	Kuhnert <i>et al.</i> (2013)
<i>H. lechatii</i>	XXX	XXX ³	MUCL 54609	French Guiana	This study
<i>H. lechatii</i>	-	XXX ²	CBS 123577	French	This study

³ sequence data deposited but no acc no obtained at the time of submission

Species	GenBank Acc.. Nos.		Strain no	Origin	Reference
	β -tubulin	ITS			
<i>H. lenormandii</i>	KC977273	KC968943	CBS 119003	Guiana (T)	Kuhnert <i>et al.</i> (2013)
<i>H. lividipigmentum</i>	AY951735	JN979433	BCRC 34077	Ecuador	Hsieh <i>et al.</i> (2005)
<i>H. monticulosum</i>	KC977302	KC968939	MUCL 54626	Mexico (IT)	Kuhnert <i>et al.</i> (2013)
<i>H. munkii</i>	KC977294	KC968912	MUCL 53315, CBS 129039	Martinique	Kuhnert <i>et al.</i> (2013)
<i>H. musceum</i>	KC977280	KC968926	MUCL 53765	Guadeloupe	Kuhnert <i>et al.</i> (2013)
<i>H. ochraceum</i>	KC977300	KC968937	MUCL 54625	Martinique	Kuhnert <i>et al.</i> (2013)
<i>H. pulicicidum</i>	JX183072	JX183075	MUCL 49879, CBS 122622	(ET)	Bills <i>et al.</i> (2012)
<i>H. rickii</i>	KC977288	KC968932	MUCL 53309, CBS 129345	Martinique	Kuhnert <i>et al.</i> (2013)
<i>H. samuelsii</i>	KC977286	KC968916	MUCL 51843	(ET)	Kuhnert <i>et al.</i> (2013)
<i>H. shearii</i> var. <i>minor</i>	AY951753	EF026142	BCRC 34093	Guadeloupe	Kuhnert <i>et al.</i> (2013)
<i>H. cf. subticinense</i>	KC977297	KC968913	MUCL 53752	(ET)	Hsieh <i>et al.</i> (2005)
<i>H. trugodes</i>	KC977284	JX658532	MUCL 49881	Mexico (IT)	Kuhnert <i>et al.</i> (2013)
				French	
				Guiana	
				Panama	Stadler <i>et al.</i> (2013b) , Kuhnert <i>et al.</i> (2013)
<i>Nemania serpens</i>	GQ470223	-	N/A	Canada	Hsieh <i>et al.</i> (2010)
	-	FN428829	CBS 533.72	Netherlands	Stadler <i>et al.</i> (2010)

^aSpecies renamed by Stadler *et al.* 2013b

Fig. 1
Metabolites of
Hypoxylon
lechatii and
related
species:

hypoxyvermelhotins A (1), B (2), C (3), vermelhotin (4), BNT (5); daldinin E (6), daldinin F (7) and macrocarpone A (8).

Fig. 2 *Hypoxylon lechatii*, from holotype specimen CLL8035. **A** Stromatal habit. **B** Stromatal surface, showing discoid areas. **C** Section through stroma showing perithecia. **D** Perithecia, showing papillate ostioles. **E** Ascospores in KOH, revealing dehiscent perispore. **F** Asci with stipes. **G** Ascospores in water, showing straight germ slit (yellow arrow). **H** Ascal tips in Melzer's reagent. **I** ex-type culture (CBS 123577) on Difco Oatmeal agar after 2 weeks on 9 cm petri dish. Scale is indicated by bars (**A**: 1 cm; **B**, **C**: 1 mm; **D**: 100 μ m; **E**, **F**, **G**, **H**: 10 μ m).

Fig. 3 Stromatal HPLC-UV profiles (200–600 nm) of holotype specimen of *Hypoxylon lechatii* and DAD spectra of major metabolites (**1** hypoxyvermelhotin A, **2** hypoxyvermelhotin B, **4** vermelhotin, **5** BNT).

Fig. 4 Phylogenetic relationships among selected *Hypoxylon* spp. and other *Xylariaceae*. Consensus tree generated by maximum likelihood (RAxML) analysis of 1000 bootstrap trees from the ITS dataset. Only bootstrap support values above 50% from 1000 replicates are indicated. Species names are followed by the GenBank accession numbers and countries of origin. The sequences of the newly described species are highlighted in grey with the type specimen labelled in bold. Type specimens are indicated by **T** (holotype), **IT** (isotype) and **ET** (epitype).

Fig. 5 Phylogenetic relationships among selected *Hypoxylon* spp. and other *Xylariaceae*. Consensus tree generated by maximum likelihood (RAxML) analysis of 1000 bootstrap trees from the β -tubulin dataset. Only the bootstrap support values above 50% from 1000 replicates are indicated. Species names are followed by the GenBank accession numbers. The sequence of the newly described species is highlighted in grey. Type specimens are indicated by T (holotype), IT (isotype) and ET (epitype).