A highly diverse spectrum of naphthoquinone derivatives produced by the endophytic fungus Biatriospora sp. CCF 4378

ARTICLE *in* FOLIA MICROBIOLOGICA · NOVEMBER 2014

Impact Factor: 1 · DOI: 10.1007/s12223-014-0366-7 · Source: PubMed

9 AUTHORS, INCLUDING:

Petr Man

Academy of Sciences of the Czech Republic

81 PUBLICATIONS **1,116** CITATIONS

SEE PROFILE

Jan Cerný

Charles University in Prague

56 PUBLICATIONS **1,889** CITATIONS

SEE PROFILE

Alena Kubátová

Charles University in Prague

55 PUBLICATIONS **585** CITATIONS

SEE PROFILE

Miroslav Kolarik

Academy of Sciences of the Czech Republic

107 PUBLICATIONS **939** CITATIONS

SEE PROFILE

Folia Microbiol
DOI 10.1007/s12223-014-0366-7

A highly diverse spectrum of naphthoquinone derivatives produced by the endophytic fungus *Biatriospora* sp. CCF 4378

Eva Stodůlková · Petr Man · Marek Kuzma · Jan Černý · Ivana Císařová · Alena Kubátová · Milada Chudíčková · Miroslav Kolařík · Miroslav Flieger

Received: 7 August 2014 / Accepted: 14 November 2014

Abstract A strain of *Biatriospora* sp. CCF 4378 was tested for the production of secondary metabolites under submerged fermentation conditions. Eleven compounds were isolated from the culture broth, and the structures of these compounds were determined using HRMS, NMR and X-ray analysis. In addition to six known naphthoquinone derivatives, i.e. ascomycone A, ascomycone B, 6-deoxyfusarubine, 6-deoxyanhydrofusarubine, herbarine and balticol A, one derivative of 2-azaanthraquinone, 6-deoxybostrycoidine, was also identified. Four new natural pyranonaphthoquinones were found, and these natural products were pleorubrin A, pleorubrin B, pleorubrin C and pleorubrin D. The toxicity on human cell lines of the crude naphthoquinone fraction and pure 6-deoxybostrycoidin, ascomycone B, pleorubrin B and 6-deoxyfusarubin was tested. Ascomycone B and 6-deoxyfusarubin elicited rapid cytotoxicity at micromolar concentrations.

Abbreviations

DMSO	Dimethyl sulfoxide
ESI	Electrospray Ionisation
HMBC	Heteronuclear multiple-bond correlation
HPLC	High-performance liquid chromatography
HRMS	High-resolution mass spectrometry
ITS	Internal transcribed spacer
LDI	Laser desorption/ionisation
LSU rDNA	Large-subunit ribosomal DNA
NMR	Nuclear magnetic resonance
PBS	Phosphate-buffered saline
SSU rDNA	Small-subunit ribosomal DNA

Introduction

Endophytic fungi living in plant tissues interact with the host and numerous co-occurring fungi and bacteria, which results in the production of many natural products with high biological activity (Qin et al. 2011; Hussain et al. 2012). An important group of endophytic fungi products is the naturally occurring pyranonaphthoquinones, which have been found in bacteria and, although less often, in plants (Thomson 1971; Sperry et al. 2008). Pyranonaphthoquinones have been shown to exhibit various biological activities, including antiviral, antibacterial and antifungal (including some pathogenic fungi) activities. These compounds also possess considerable pharmaceutical potential, and the antibiotic, antitumor, insecticidal phytotoxic effects of these compounds have been repeatedly demonstrated (Sperry et al. 2008; Brimble et al. 1999). Isofuranonaphthoquinones were determined as products of several fungi, including *Nectria haematococca* (Parisot et al. 1983), *Fusarium oxysporum* (Tatum et al. 1987; Bell et al. 2003), *Arthrinium* sp. (Qiancutrone et al. 1994), *Cercophora*

Electronic supplementary material The online version of this article (doi:10.1007/s12223-014-0366-7) contains supplementary material, which is available to authorized users.

E. Stodůlková · P. Man · M. Kuzma · M. Chudíčková · M. Kolařík · M. Flieger (✉)
Institute of Microbiology of the ASCR, v.v.i., Vídeňská 1083, 142 20 Prague 4, Czech Republic
e-mail: flieger@biomed.cas.cz
URL: http://www.biomed.cas.cz/mbu/lab112/people.html

J. Černý
Department of Cell Biology, Faculty of Science, Charles University, 12840 Prague, Czech Republic

I. Císařová
Department of Inorganic Chemistry, Faculty of Science, Charles University, 12840 Prague 2, Czech Republic

A. Kubátová · M. Kolařík
Department of Botany, Faculty of Science, Charles University, Benátská 2, 12801 Prague 2, Czech Republic

Published online: 22 November 2014

sordarioides (Whyte et al. 1997) and *Monosporascus cannonballus* (Stipanovic et al. 2004), and the biological activities of these compounds include antibacterial and antifungal effects and limited cytotoxicity (Piggott 2005). Furanonaphthoquinones cause the apoptosis of cancer cells by inducing the production of reactive oxygen species (Simamura et al. 2006). The acyl substituted isofuranonaphthoquinones were isolated from the mycobiont of the lichen *Arthonia cinnabarina* (Yamamoto et al. 2002). 2-Azaanthraquinones are primarily produced by fungi or lichens and also display various antimicrobial activities (Van Wagoner et al. 2008; Koyama et al. 2005; Baker et al. 1990; Schüffler et al. 2009b).

During our mycological survey of the phloem endophytes of *Ulmus laevis* (European white elm), we isolated a sterile fungus that was identified as an undescribed species with endophytic/marine aquatic lifestyle related to *Biatriospora marina* (Ascomycota: Pleosporales). Among all the isolated fungi, this species showed a unique antifungal effect against *Pyronema domesticum*, a highly competitive and rapidly growing fungus that overgrows primary isolation plates with other endophytic fungi. In the present study, we provide a comprehensive analysis of the natural products produced by *Biatriospora* sp. CCF 4378 and report on four new and seven previously known metabolites.

Material and methods

Fungal isolation A healthy twig of *U. laevis* was collected in 2008 from Libicky Luh Forest near Velky Osek, Czech Republic. The fungal endophyte was isolated from the surface of sterilised phloem slivers using the methods of Pažoutová et al. (2012). The strain CCF 4378 (AK165/08) is maintained in the Culture Collection of Fungi, Charles University in Prague.

Identification of fungal culture The fungus, morphologically characterised as a dark sterile mycelium, was identified using DNA sequencing. Using previously published procedures, the nuclear internal transcribed spacer (ITS) region together with partial large-subunit (LSU) and small-subunit (SSU) ribosomal DNA (rDNA) was amplified with the primer set ITS1F/LR6 and NS1/NS4. Additionally, the partial β-tubulin gene sequence was amplified with primers T1/T2, and the partial sequence of the gene for elongation factor 1α was amplified using the primers EF1-728 F/EF1-986R (Pažoutová et al. 2012; Kolařík and Jankowiak 2013). The EMBL accession numbers of the sequence data are JX570932, JX570934 and JX570933. Our amplificates were compared with the published sequences using a Blast similarity search in the GenBank NCBI database (Altschul et al. 1997). Phylogenetic analysis comparing our sequence and the best matches from GenBank was conducted in PhyMLOnline (http://atgc.lirmm.fr/phyml/). The detailed results are provided in the supplementary material. The ITS rDNA sequence was identical to several GenBank entries and was obtained from undetermined sterile endophytic or marine fungi. LSU and SSU rDNA showed the best match (99 %) with *Nigrograna mackinnonii* and *B. marina*. The protein-coding genes confirmed this taxonomic position, and the closest matches (94 %) belonged to *B. marina* and other members of Pleosporales. Based on the ITS, LSU and SSU rDNA phylogenetic analysis, our fungus belongs to the clade of *Nigrograna mackinnonii* and *B. marina*, which was formerly recognised as a separate lineage of Pleosporales (Suetrong et al. 2009). We decided to use the generic name *Biatriospora*, which is the older in the whole group. Our analysis clearly indicates that the studied fungus belongs to fungi with the potential to be an endophyte of terrestrial plants as well as marine aquatic fungus.

Cultivation The fungus was cultivated on a malt extract medium containing (g/L) malt extract, 20; glucose, 20; peptone, 1. The submerged cultivations were performed in 500-mL Erlenmeyer flasks containing 50 mL of media (5 L in total) on a rotary shaker (3.4 Hz) for the period of 14 days at 24 °C in the dark.

Extraction and fractionation Fermentation broth (4 L) of *Biatriospora* sp. CCF 4378 was centrifuged and extracted three times with an equal volume of CH_2Cl_2. The pooled extracts were dried over anhydrous Na_2SO_4, filtered and evaporated to dryness under reduced pressure. The crude extract (720 mg) was further subjected to reversed phase flash chromatography (VersaPak, 40×150 mm, Supelco, Bellefonte, PA, USA) and eluted with a stepwise (10 %) gradient of MeOH in H_2O (0–100 %). Naphthoquinone derivatives were found only in fractions eluted with 40 (fraction 1), 50 (fraction 2), 60 (fraction 3) and 70 % of methanol (fraction 4), respectively. These fractions were partially evaporated and further analysed by HPLC.

HPLC The HPLC system (Waters, Milford, MA, USA) consisted of a pump equipped with a 600E system controller, autosampler 717 and dual UV detector 2487. The data were processed with the Empower 2 software. Water-containing mobile phases were filtered through a 0.22-μM GS filter (Millipore, Billerica, MA, USA) and degassed in an ultrasonic bath for 10 min before use. UV detection was performed at 275 and 350 nm.

A Gemini 5-μm C18 column (250×4.6 mm, Phenomenex) with a guard column was used for the preparation of standards. The mobile phase consisted of water (A) and methanol (B). Gradient elution started at 2 % B (0 min), increasing linearly to 100 % B within 40 min, at a flow rate of 1.0 mL/min.

Repeated HPLC analysis of the fraction 1 followed by mass spectrometry (MS) and NMR analysis of the individual compounds resulted in the collection of three naphthoquinone derivatives, i.e. balticol A (retention time 2.8 min, yield 4.5 mg), pleorubrin B (4.0 min, 10.2 mg) and 6-deoxyfusarubin (5.8 min, 12.5 mg). The same procedure was applied for all other fractions, and the following compounds were isolated: fraction 2—ascomycone B (7.7 min, 17 mg), herbarine (8.6 min, 3.2 mg) and pleorubrin C (9.1 min, 2.6 mg); fraction 3—6-deoxybostrycoidine (10.5, 2.3 mg), pleorubrin A (11.0 min, 1.8 mg), pleorubrin D (11.6 min, 1.1 mg) and ascomycone A (12.8 min, 8.1 mg); and fraction 4 contains residual amount of ascomycone A and 6-deoxyanhydrofusarubine (13.5 min, 1.5 mg).

Mass spectrometry MS experiments were performed on a commercial 9.4 T APEX-Ultra FTMS instrument (Bruker Daltonics, Billerica, MA, USA) equipped with an electrospray ionisation (ESI)/matrix-assisted laser desorption/ionisation (MALDI) ion source. The instrument operated in both positive and negative ion modes. Spectra were collected over the mass range of 150–2000 m/z at 1 M data points resulting in a maximum resolution of 200,000 at m/z 400. One microlitre of each sample (1 mg/mL) was dissolved in 0.1 mL of MeOH/water 1:1 and introduced to the MS by direct infusion via ESI source and/or spotted on a MALDI plate. The instrument was externally calibrated using singly charged arginine clusters, resulting in sub-ppm accuracy. The spectra were apodised using sin apodisation with one zero fill. Data were processed in Data Analysis and the possible elemental compositions were calculated using Smart Formula calculations.

NMR spectrometry NMR spectra were recorded on a Bruker Avance III 600-MHz spectrometer (600.23 MHz for ^{1}H, 150.93 MHz for ^{13}C) in CD_3CN (at 30 °C) and CD_2Cl_2 (at 20 °C) (99.8 atom% D, SIGMA-ALDRICH, Steinheim, Germany). The residual signal of the solvent was used as an internal standard (CD_3CN, δ_H 1.930 ppm, δ_C 1.39 ppm; CD_2Cl_2, δ_H 5.320 ppm, δ_C 54.00 ppm). In NMR experiments, ^{1}H NMR, ^{13}C NMR, J-resolved, COSY, ^{1}H-^{13}C HSQC, ^{1}H-^{13}C heteronuclear multiple-bond correlation (HMBC), and ^{1}H-^{15}N HMBC were performed using the manufacturer's software. ^{1}H NMR and ^{13}C NMR spectra were zero filled to fourfold data points and multiplied by the window function before the Fourier transformation. A two-parameter double-exponential Lorentz-Gauss function was applied for ^{1}H spectra to improve the resolution, and line broadening (1 Hz) was applied to obtain a better ^{13}C signal-to-noise ratio. Chemical shifts are given in the δ-scale with digital resolution justifying the reported values to three (δ_H) or two (δ_C) decimal places.

X-ray Single-crystal X-ray diffraction data for pleorubrin C and balticol A were obtained from a Nonius KappaCCD diffractometer equipped with a Bruker ApexII detector by monochromatised MoKα radiation (λ=0.71073 Å) at 150(2)K. The structures were solved by direct methods (SHELXS, Sheldrick 2008) and refined by full-matrix least squares based on F2 (SHELXL97). The hydrogen atoms on carbons were fixed into idealised positions (riding model) and assigned with temperature factor Hiso (H)=1.2 Ueq (pivot atom). The hydrogen atom of the hydroxyl was found on the difference Fourier map and refined isotropically.

Biological activity Adenocarcinoma HeLa cells and primary human skin fibroblasts were cultivated in D-MEM medium supplemented with 10 % FCS (Gibco, Invitrogen, Carlsbad, CA, USA) and grown on glass cover slips (up to 50 % density) in six-well plates (Nunc, Thermo Fisher Scientific, Waltham, MA, USA) treated with compounds dissolved in DMSO (stock solution 10 mM) for various times and concentrations. Wells containing DMSO only (max 1 %) were assayed as a blank control—no alterations of the cellular morphology and physiology were observed. All the samples were tested in triplicate. The treated cells (various times and concentrations) grown at 37 °C in a humidified 5 % CO_2 atmosphere were treated with fluorescent trackers (LIVE/DEAD® Viability/Cytotoxicity Kit, Holotransferrin Alexa Fluor®594 conjugate or MitoTracker® Red CMXRos) or fixed (3.7 % paraformaldehyde in PBS, 20 min, RT), permeabilised (0.1 % Triton X-100 in PBS), blocked (1 % BSA in PBS) and stained with Phalloidin-Alexa Fluor®488 conjugate. Staining of the nuclei was performed by mounting the specimens in Mowiol-DAPI or in vivo in cells with permeable plasma membranes by diethidium bromide. All the fluorescent reagents were purchased from Molecular Probes, Invitrogen, Carlsbad, CA, USA.

Results and discussion

Fermentation and isolation of individual compounds Strain isolation, characterisation, fermentation conditions and individual compound isolation are given in the "Material and methods" section. Briefly, centrifuged culture broth was repeatedly extracted with dichloromethane, and individual compounds were isolated using different chromatographic techniques. Final purification was performed by HPLC. These processes provided 11 compounds.

Mass spectrometry The individual fractions from HPLC were diluted with methanol/water and directly spotted on a MALDI plate or infused into the FT-ICR mass spectrometer via an electrospray source. The samples were analysed in both modes, positive and negative. A majority of the studied compounds gave clear signals when subjected to direct LDI. This

behaviour can be attributed to the aromatic nature of these compounds.

However, in some cases, better results were observed in the ESI spectra. These cases especially apply to the tetrahydrogenated forms of pyranonaphthoquinones that gave no signal in the LDI mode, and the mass spectra were recorded only using electrospray ionisation (Table 1). While the ESI spectra were dominated by protonated (positive ion mode) and deprotonated species (negative ion mode), the LDI spectra provided M+H species in the positive ion mode and M^- species in the negative ion mode. Here, small peaks corresponding to the deprotonated forms can be observed for all the compounds providing negative ion mode LDI spectra.

NMR spectrometry The structures of the isolated naphthoquinone derivatives were further elucidated by NMR spectrometry. The naphthoquinone parts of all the compounds (with the exception of pleorubrin B) exhibit an aromatic AB spin system of protons with a *meta* relationship in the ^{1}H NMR spectrum. This spin system is due to the determined coupling constant ^4J=2.5 Hz. Further, a singlet of the CH_3O- group and the down-field resonating HO- group were also observed. The ^{13}C NMR spectrum contains two down-field resonating carbonyls. The remaining quaternary carbons were identified by ^{1}H-^{13}C HMBC correlation (Fig. 1). Because the isolated compounds differ in the C ring (with the exception of pleorubrin B), the elucidation of the structure of the individual compounds focuses on this part.

This procedure led to the determination of the structure of seven known compounds, i.e. 6-deoxyanhydrofusarubin, ascomycone A, ascomycone B, 6-deoxyfusarubin, herbarin, balticol A and 6-deoxybostrycoidin. In all the cases, the obtained NMR spectra were in agreement with the previously published data (Parisot et al. 1989, 1992; Opatz et al. 2008; Paranagama et al. 2007; Narasimh and Gopalkri 1974; Jadulco et al. 2002; Schüffler et al. 2009a; Shushni et al. 2009).

Structural characterisation of individual compounds

Pleorubrin A

The LDI FTMS spectrum of pleorubrin A reveals the molecular formula $C_{15}H_{14}O_5$ with a $[M]^-$ peak at m/z=274.08443. The C ring constitutes an isolated oxymethylene and the $CH_3CH(O)CH_2$- spin system. The observed HMBC correlation between H-3 and C-1 confirms the $–CH_2$-CH(CH_3)-O-CH_2- moiety. The protons of the C-1 methylene group have HMBC contact with the C-10 carbonyl C-10. The second carbonyl (position 5) is coupled in HMBC to the C-4 protons. The value of ^3J(H-3,H-4 eq)=10.0 Hz indicates a pseudo-equatorial orientation of the 3-CH_3 group. This fact together with the determined specific rotation $[\alpha]_D^{20}$+126.0 (c 1.34, $CHCl_3$) (lit. +60.5, Sperry et al. 2009) allows us to determine the structure of pleorubrin A as (S)-9-hydroxy-7-methoxy-3-methyl-3,4-dihydro-1*H*-benzo[g]isochromene-5,10-dione (Fig. 2, structure 1). This compound was previously synthesised by Sperry et al. (2009).

^{13}C NMR (150.93 MHz, CD_3CN, 30 °C) δ 21.49 (q, 3-CH_3), 30.31 (t, C-4), 57.15 (q, 7-OCH_3), 63.56 (t, C-1), 70.39 (d, C-3), 106.74 (d, C-8), 108.57 (d, C-6), 110.24 (s, C-9a), 134.90 (s, C-5a), 143.56 (s, C-4a), 143.87 (s, C-10a), 165.26 (s, C-9), 167.26 (s, C-7), 183.89 (s, C-5), 188.30 (s, C-10).

^{1}H NMR (600.23 MHz, CD_3CN, 30 °C) δ 1.297 (3H, d, *J*=6.2 Hz, 3-CH_3), 2.165 (1H, dddd, *J*=2.7, 4.2, 10.0, 18.9 Hz,

Table 1 Mass spectrometric data of naphthoqiunone derivatives isolated from submerged culture of *Biatriospora* sp. CCF 4378

Compound	Elemental composition	Theoretical mass	Measured mass (m/z)			
			LDI+	LDI−	ESI+	ESI−
6-Deoxyanhydrofusarubin	$C_{15}H_{12}O_5$	272.06846	273.07549	272.06881	273.07642	nd
Asocomycone A	$C_{16}H_{14}O_6$	302.07902	nd	302.07931	nd	nd
Asocomycone B	$C_{15}H_{11}O_6$	287.05555	271.0598 [M-H_2O]	287.05614	271.0610 [M-H_2O]	nd
6-Deoxyfusarubin	$C_{15}H_{14}O_6$	290.07902	nd	290.07954	291.08694	289.06836
Herbarin	$C_{16}H_{16}O_6$	304.09467	305.10212	304.09504	305.10278	303.08745
Pleorubrin A	$C_{15}H_{14}O_5$	274.08411	nd	274.08443	nd	nd
Pleorubrin B	$C_{15}H_{18}O_5$	278.11541	nd	nd	279.12322	277.10822
Balticol A	$C_{14}H_{16}O_5$	264.09976	nd	nd	nd	263.09251
Pleorubrin C	$C_{15}H_{10}O_6$	286.04773	287.05514	286.04817 $[M]^-$ 285.04051 $[M-H]^-$	287.05573	285.04088
Pleorubrin D	$C_{14}H_{10}O_5$	258.05281	259.06042	258.05329	259.06067	nd
6-Deoxybostrycoidin	$C_{15}H_{11}O_4N$	269.06880	270.07635	269.06929 $[M]^-$	270.0766	nd

nd not detected

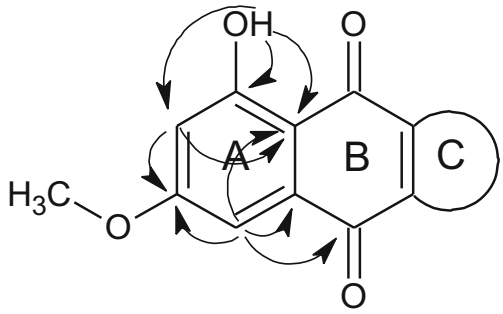


Fig. 1 The partial structure of the studied compounds with key HMBC correlations of the naphthoquinone part

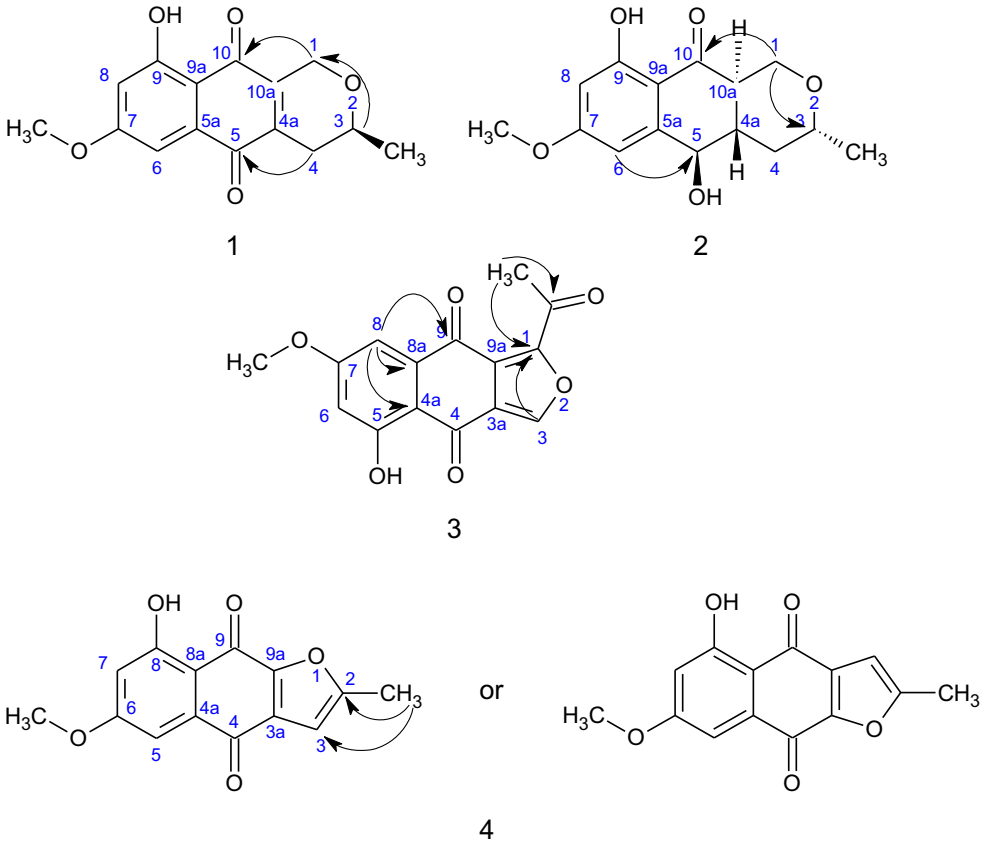
H-4 eq), 2.654 (1H, dddd, *J*=0.9, 3.2, 3.2, 18.9 Hz, H-4ax), 3.655 (1H, dqd, *J*=3.2, 6.2, 10.0 Hz, H-3), 3.892 (3H, s, 7-OCH_3), 4.442 (1H, ddd, *J*=3.2, 4.2, 18.5 Hz, H-1 eq), 4.732 (1H, ddd, *J*=0.9, 2.7, 18.5 Hz, H-1ax), 6.688 (1H, d, *J*= 2.5 Hz, H-8), 7.104 (1H, d, *J*=2.5 Hz, H-6), 12.064 (1H, s, 9-OH).

Pleorubrin B

The molecular formula is $C_{15}H_{18}O_5$, and this formula can be estimated based on the $[M+H]^+$ ion at m/z 279.12322. Pleorubrin B substantially differs from all the other substances. The ^{1}H NMR spectrum contains two singlets of methoxyl and hydroxyl groups and two isolated spin systems, one AB spin system of aromatic protons with a *meta* orientation and a spin system of the –$CH(OH)CH(CHCH_2O)CH_2CHCH_3$ type. The HMBC correlations allowed us to identify aromatic spin systems substituted by methoxyl and hydroxyl groups. One carbonyl in the *peri*-position was also detected. Furthermore, the HMBC correlations between H-6 and C-5 and the protons at C-1 to C-3 and C-10 were also observed. Based on these observations, the structure of pleorubrin B was elucidated as 5,9-dihydroxy-7-methoxy-3-methyl-1,3,4,4a,5,10a-hexahydro-1*H*-benzo[g]isochromen-10-one (Fig. 2, structure 2) which agrees with the ESI FT-MS spectrum containing the pseudomolecular ion $[M+H]^+$ at m/z 279.12322 indicating the molecular formula $C_{15}H_{18}O_5$. The set of coupling constants of the C ring agrees well with the chair conformation. The coupling constant between the protons H-4a and H-10 a ^3J=12.5 Hz indicates the *trans*-arrangement of these protons. The value of ^3J(H-3,H-4 eq)=11.1 Hz indicates an equatorial orientation of the 3-CH_3 group. These conclusions were also verified by preliminary data obtained by X-ray analysis (Fig. 3).

^{13}C *NMR* (150.93 MHz, CD_3CN, 30 °C) δ 22.23 (q, 3-CH_3), 37.44 (t, C-4), 46.26 (d, C-4a), 48.20 (d, C-10a), 56.59 (q, 7-OCH_3), 67.07 (t, C-1), 72.47 (d, C-5), 73.85 (d, C-3), 100.59

Fig. 2 Structures of new pyranonaphthoquinones: **1** pleorubrin A, **2** pleorubrin B, **3** pleorubrin C, and **4** two alternative structures of pleorubrin D



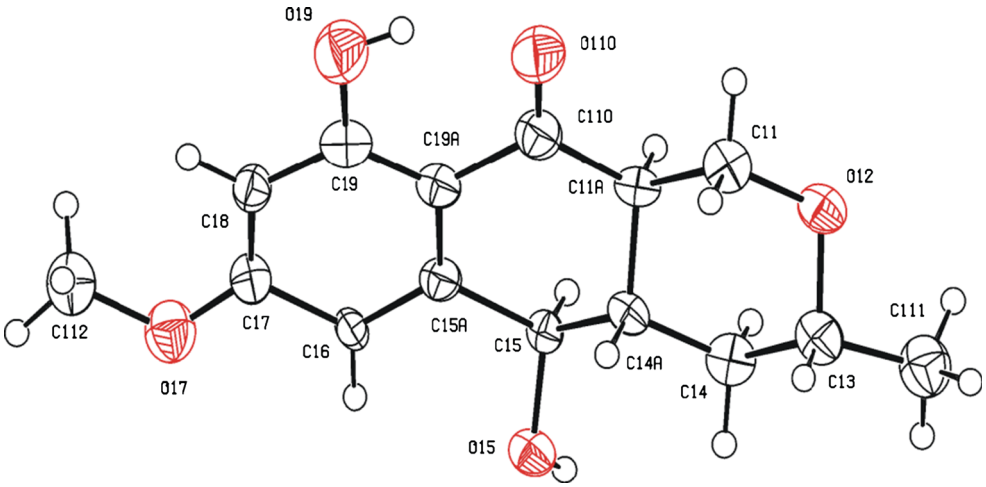


Fig. 3 The X-ray preliminary structure of pleorubrin B

(d, C-8), 105.25 (d, C-6), 110.68 (s, C-9a), 151.78 (s, C-5a), 166.32 (s, C-9), 167.65 (s, C-7), 202.63 (s, C-10).

^{1}H NMR (600.23 MHz, CD_3CN, 30 °C) δ 1.172 (1H, ddd, *J*= 11.0, 11.5, 13.1 Hz, H-4 eq), 1.182 (1H, d, *J*=6.2 Hz, 3-CH_3), 2.009 (1H, dddd, *J*=3.9, 10.6, 11.5, 12.5 Hz, H-4a), 2.142 (1H, ddd, *J*=2.0, 3.9, 13.1 Hz, H-4ax), 2.536 (1H, ddd, *J*=4.6, 10.3, 12.5 Hz, H-10a), 3.411 (1H, dd, *J*=10.3, 11.5 Hz, H-1 eq), 3.413 (1H, dqd, *J*=2.0, 6.2, 11.0 Hz, H-3), 3.653 (1H, d, *J*=7.6 Hz, 5-OH), 3.838 (3H, s, 7-OCH_3), 4.377 (1H, dd, *J*=4.6, 11.5 Hz, H-1ax), 4.427 (1H, dddd, *J*=0.7, 1.3, 7.6, 10.6 Hz, H-5), 6.331 (1H, dd, *J*=0.7, 2.5 Hz, H-8), 6.730 (1H, dd, *J*=1.3, 2.5 Hz, H-6), 12.596 (1H, s, 9-OH),

Pleorubrin C

The molecular formula is $C_{15}H_{10}O_6$, and this formula can be estimated based on the $[M+H]^+$ ion at m/z 287.05514. The additional ^{1}H NMR signals (C ring constituents) is from one aromatic methine and one methyl group. The methyl group has HMBC correlations to a carbonyl and quaternary carbon at 152.10 ppm. The chemical shift of C-3 agrees well with the attachment of this carbon to an oxygen atom. Furthermore, the proton H-8 exhibits HMBC correlations to the carbonyl C-9 and two quaternary aromatic carbons. All the NMR data of pleorubrin C are consistent with the structure of 1-acetyl-5-hydroxy-7-methoxynaphtho[2,3-c]furan-4,9-dione (Fig. 2, structure 3).

^{13}C NMR (150.93 MHz, CD_2Cl_2, 20 °C) δ 29.64 (q, CH_3), 56.82 (q, 7-OCH_3), 106.90 (d, C-6), 109.58 (d, C-8), 111.76 (s, C-4a), 122.86, 125.35 (s, C-3a, C-9a), 138.03 (s, C-8a), 147.16 (d, C-3), 152.10 (s, C-1), 166.47 (s, C-5), 167.32 (s, C-7), 178.16 (s, C-9), 183.74 (s, C-4), 187.51 (s, 1-C=O).

^{1}H NMR (600.23 MHz, CD_2Cl_2, 20 °C) δ 2.808 (3H, s, CH_3), 3.942 (3H, s, 7-OCH_3), 6.727 (1H, d, *J*=2.5 Hz, H-6), 7.378 (1H, d, *J*=2.5 Hz, H-8), 8.319 (1H, s, H-3), 12.671 (1H, s, 5-OH).

X-ray Crystal data for pleorubrin C: $C_{15}H_{10}O_6$, M_r=286.23, Monoclinic, *P* $2_1/c$ (No 14), *a*=7.8111 (3) Å, *b*=21.8179 (8) Å, *c*=7.2243 (3) Å, β=101.675 (2)°, *V*=1205.71 (8) $Å^3$, Z=4, D_x=1.577 Mg m^{-3}, yellow crystal of dimensions 0.89×0.42× 0.07 mm, multi-scan absorption correction (μ=0.12 mm^{-1}) T_{min}=0.8980, T_{max}=0.9919; a total of 14,737 measured reflections (θ_{max}=27.5°), from which 2757 were unique (R_{int}= 0.028) and 2136 were observed according to the $I>2\sigma(I)$ criterion. The refinement converged ($\Delta/\sigma_{max}<0.001$) to *R*= 0.037 for the observed reflections and $wR(F^2)$=0.104, *GOF*= 1.03 for 196 parameters and all 2757 reflections. The final difference map displayed no peaks of chemical significance ($\Delta\rho_{max}$=0.33, $\Delta\rho_{min}$ −0.22 e.$Å^{-3}$) (Fig. 4).

CCDC-974295 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/ data_request/cif.

Pleorubrin D

The measured $[M+H]^+$ ion at m/z 259.06042 for pleorubrin D is consistent with the molecular formula $C_{14}H_{10}O_5$. The C ring of this compound is composed of olefinic methine and methyl with a double bond. The methyl group is coupled to the quaternary carbon and methine forming the –CH=C(CH_3)- group. The chemical shift of the quaternary carbon (162.32 ppm) agrees well with the oxygen substitution. The missing HMBC correlations of the proton at C-3 to any carbonyl prevented unequivocally arranging the ring C to the rest of the molecule. Therefore, these two possibilities are depicted in Fig. 2 (structures 4).

^{13}C NMR (150.93 MHz, CD_3CN, 30 °C) δ 14.15 (q, 2-CH_3), 57.22 (q, 6-OCH_3), 105.88 (d, C-3), 106.90 (d, C-7), 110.01

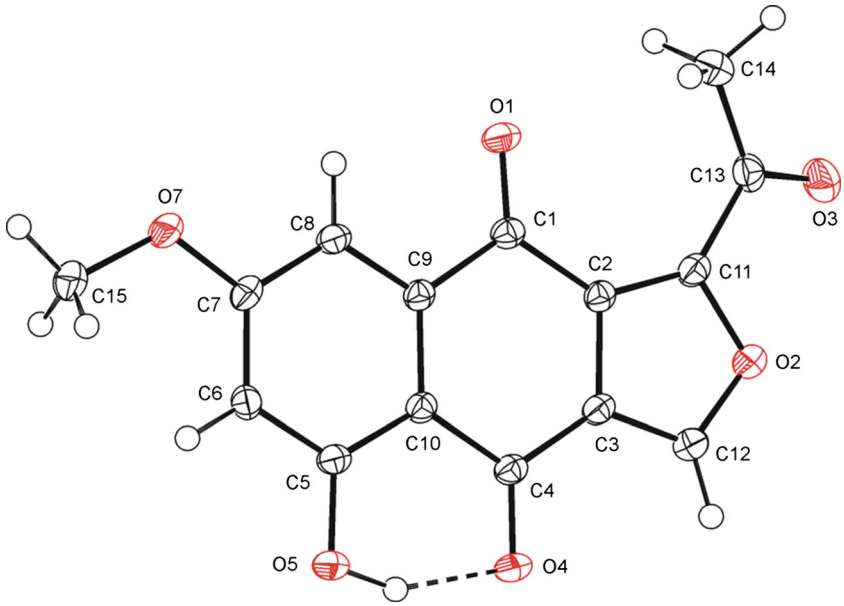


Fig. 4 The X-ray structure of pleorubrin C

(d, C-5), 110.1* (s, C-8a), 133.18 (s, C-3a), 136.44 (s, C-4a), 152.58 (s, C-9a), 162.32 (s, C-2), 166.26 (s, C-8), 167.15 (s, C-6), 178.28 (s, C-9), 180.74 (s, C-4). (* HMBC readout)

^1H NMR (600.23 MHz, CD_3CN, 30 °C) 2.473 (d, *J*=1.0 Hz, 2-CH_3), 3.902 (s, 6-OCH_3), 6.613 (q, *J*=1.0 Hz, H-3), 6.700 (d, *J*=2.5 Hz, H-7), 7.188 (d, *J*=2.5 Hz, H-5), 12.250 (s, 8-OH).

Compound toxicity and the effect on actin cytoskeleton of human cell lines

The crude naphthoquinone fraction containing a mixture of all identified naphthoquinones was tested on adenocarcinoma HeLa cells. Obvious bioactivity (a lack of adherence and reorganisation of the actin cytoskeleton) and toxicity (positivity of the nuclei for cell impermeable nuclear dyes) were observed (from 10 μg/mL, 12-h incubation). Similar results were obtained using primary human skin fibroblasts (Fig. 5).

Pyranonaphthoquinones obtained in sufficient quantities (e.g. 6-deoxybostrycoidin, ascomycone B, pleorubrin B and 6-deoxyfusarubin) were tested individually. Surprisingly, compounds with similarities in chemical structure elicited different bioactivities ranging from no effect (6-deoxybostrycoidin, pleorubrin B—up to 100 μg/mL) to dramatic changes of the cellular content and cell death (6-deoxyfusarubin, ascomycone B). The effect was rapid (in minutes) in both the cell types tested (HeLa cells and primary human skin fibroblasts). During longer incubation times, (24 h, 50 μg/mL) all cells died via necrotic cell death (Fig. 6).

The effect at microgram concentrations starts with a change in mitochondrial organisation (from a filamentous network to individual perinuclear vesicles; data not shown) and is followed by a reorganisation of the actin cytoskeleton cellular filopodialisation, rounding, and blocked transferrin uptake and ends with a lack of detectable esterase and nucleic acid content.

In conclusion, our strain belongs to the undescribed *Biatriospora* species, and members of this species have been found in the Baltic sea (drifting wood) (Shushni et al. 2009)

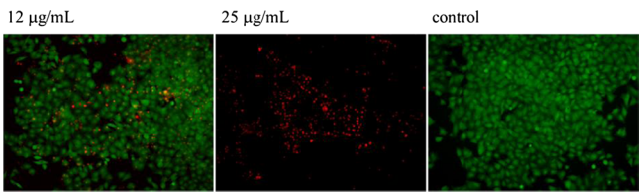


Fig. 5 HeLa cells treated with the crude naphthoquinone fraction and tested with the LIVE/DEAD® Viability/Cytotoxicity Kit (*red* ethidium homodimer positive nuclei indicate enhanced membrane permeability; the *green signal* indicates the viability of the cells)

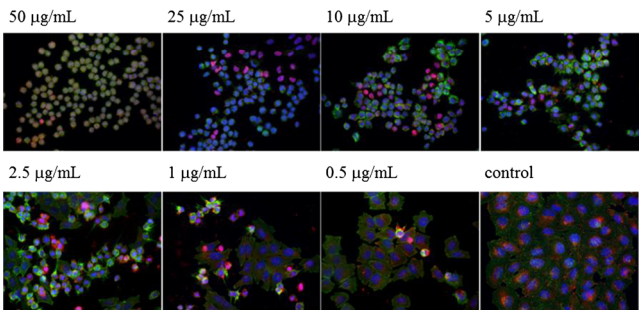


Fig. 6 Activity of 6-deoxyfusarubin on HeLa cells (24 h, 20 min in vivo incubation with fluorescently labelled transferrin—*red* perinuclear endosomal pattern and ethidium homodimer—red nuclear signal in dying/dead cells (*top panel*); after fixation, actin cytoskeleton was visualised using fluorescently labelled phalloidin—*green signal*, nuclei were labelled with DAPI blue)

and in Italy in the roots of marine and litoral plants (Panno et al. 2013; Angelini et al. 2012) (see Supporting information). The isolate from the drifting wood is known as a producer of numerous biologically active compounds (Shushni et al. 2009, 2011, 2013) found in our study, including balticol A, 5-deoxyanhydrofusarubin and 5-deoxybostrycoidine. In addition, a diverse group of furanonaphthoquinones was detected. The studied strain produced pyranonaphthoquinones known in other endophytic or marine members of the fungal order Pleosporales such as ascomycones (Opatz et al. 2008), which have been isolated from closely related species (see Supporting information), and herbarin (Paranagama et al. 2007; Jadulco et al. 2002; Schüffler et al. 2009b). The taxonomically related marine fungus *Amorosia littoralis* is known to produce 2-azaanthraquinone (Van Wagoner et al. 2008). Phylogenetic analysis placed our fungus in a lineage comprising endophytic as well as marine aquatic fungi. Both ecological groups are known as potent producers of biologically active natural products. Our studies proved the presence of highly active substances and illustrated that the isolate has strong potential against the aggressive, competitive and rapidly growing fungus *P. domesticum*. A cocktail of the bioactive compounds produced by the isolate can not only limit the growth of fungi and bacteria but also alter the physiology of human cell lines.

Acknowledgments This work was supported by the LD-COST CZ project LD13039, Czech Science Foundation project No. 13-16565S, Long-Term Research Plans of the Ministry of Education, Youth and Sports of the Czech Republic No. MSM0021620858, and Charles University projects UNCE 204013/2012. Access to instrumental and other facilities was also supported by the EU (Operational Program Prague–Competitiveness project CZ.2.16/3.1.00/24023) and by the IMIC institutional research concept RVO61388971.

References

Altschul SF, Madden TL, Schaffer AA, Zhang JH, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389–3402

Angelini P, Rubini A, Gigante D, Reale L, Pagiotti R, Venanzoni R (2012) The endophytic fungal communities associated with the leaves and roots of the common reed (*Phragmites australis*) in Lake Trasimeno (Perugia, Italy) in declining and healthy stands. Fungal Ecol 5:683–693

Baker RA, Tatum JH, Nemec S (1990) Antimicrobial activity of naphthoquinones from fusaria. Mycopathologia 111:9–15

Bell AA, Wheeler MH, Liu JG, Stipanovic RD (2003) United States Department of Agriculture—Agricultural Research Service studies on polyketide toxins of *Fusarium oxysporum* f. sp. *vasinfectum*: potential targets for disease control. Pest Manag Sci 59:736–747

Brimble MA, Duncalf LJ, Nairn MR (1999) Pyranonaphthoquinone antibiotics—isolation, structure and biological activity. Nat Prod Rep 16:267–281

Hussain H, Krohn K, Ahmed I, Draeger S, Schulz B, Di Pietro S, Pescitelli G (2012) Phomopsinones A-D: four new pyrenocines from endophytic fungus *Phomopsis* sp. Eur J Org Chem:1783–1789

Jadulco R, Brauers G, Edrada RA, Ebel R, Wray V, Sudarsono PP (2002) New metabolites from sponge-derived fungi *Curvularia lunata* and *Cladosporium herbarum*. J Nat Prod 65:730–733

Kolařík M, Jankowiak R (2013) Vector affinity and diversity of Geosmithia fungi living on subcortical insects inhabiting *Pinaceae* species in central and northeastern Europe. Microb Ecol 66:682–700

Koyama J, Morita I, Kobayashi N, Osakai T, Usuki Y, Taniguchi M (2005) Structure-activity relations of azafluorenone and azaanthraquinone as antimicrobial compounds. Bioorg Med Chem Lett 15:1079–1082

Narasimh N, Gopalkri KS (1974) Naphthoquinone pigments from *Torula herbarum*—structure of methylherbarin. J Antibiot 27:283–287

Opatz T, Kolshorn H, Thines E, Anke H (2008) Ascomycones A-C, heptaketide metabolites from an unidentified ascomycete. J Nat Prod 71:1973–1976

Panno L, Bruno M, Voyron S, Anastasi A, Gnavi G, Miserere L, Varese GC (2013) Diversity, ecological role and potential biotechnological applications of marine fungi associated to the seagrass *Posidonia oceanica*. New Biotechnol 30:685–694

Paranagama PA, Wijeratne EMK, Burns AM, Marron MT, Gunatilaka MK, Arnold AE, Gunatilaka AAL (2007) Heptaketides from *Corynespora* sp. inhabiting the cavern beard lichen, *Usnea cavernosa*: first report of metabolites of an endolichenic fungus. J Nat Prod 70:1700–1705

Parisot D, Devys M, Ferezou JP, Barbier M (1983) Pigments from *Nectria haematococca*, anhydrofusarubin and nectriafurone. Phytochemistry 22:1301–1303

Parisot D, Devys M, Barbier M (1989) 5-Deoxybostrycoidin, a new metabolite produced by the fungus *Necteria haematococca* (Berk and Br) WR. Z Naturforsch B J Chem Sci 44:1473–1474

Parisot D, Devys M, Barbier M (1992) Heptaketide derived polyenes from the fungus *Necteria haematococca*. Phytochemistry 31:4357–4358

Pažoutová S, Šrůtka P, Holuša J, Chudíčková M, Kubátová A, Kolařík M (2012) *Liberomyces* gen. nov. with two new species of endophytic coelomycetes from broadleaf trees. Mycologia 104:198–210

Piggott MJ (2005) Naphtho[2,3-c]furan-4,9-diones and related compounds: theoretically interesting and bioactive natural and synthetic products. Tetrahedron 61:9929–9954

Qiancutrone JF, Gao Q, Huang S, Klohr SE, Veitch JA, Shu YZ (1994) Arthrinone, a novel fungal metabolite from *Arthrinium* sp. FA-1744. J Nat Prod 57:1656–1660

Qin S, Krohn K, Hussain H, Schulz B, Draeger S (2011) Pestalotheols E-H: antimicrobial metabolites from an endophytic fungus isolated from the tree *Arbutus unedo*. Eur J Org Chem: 5163–5166

Schüffler A, Liermann JC, Kolshorn H, Opatz T, Anke H (2009a) Isolation, structure elucidation, and biological evaluation of the unusual heterodimer chrysoxanthone from the ascomycete IBWF11-95A. Tetrahedron Lett 50:4813–4815

Schüffler A, Liermann JC, Kolshorn H, Opatz T, Anke H (2009b) New naphthoquinone derivatives from the ascomycete IBWF79B-90A. Z Naturforsch C Biosci 64:25–31

Sheldrick GM (2008) A short history of SHELX. Acta Crystallogr A 64: 112–122

Shushni MAM, Mentel R, Lindequist U, Jansen R (2009) Balticols A-F, new naphthalenone derivatives with antiviral activity, from an ascomycetous fungus. Chem Biodivers 6:127–137

Shushni MAM, Singh R, Mentel R, Lindequist U (2011) Balticolid: a new 12-membered macrolide with antiviral activity from an ascomycetous fungus of marine origin. Mar Drugs 9:844–851

Shushni MAM, Azam F, Lindequist U (2013) Oxasetin from *Lophiostoma* sp of the Baltic sea: identification, *in silico* binding

mode prediction and antibacterial evaluation against fish pathogenic bacteria. Nat Prod Comm 8:1223–1226

Simamura E, Hirai KI, Shimada H, Koyama J, Niwa Y, Shimizu S (2006) Furanonaphthoquinones cause apoptosis of cancer cells by inducing the production of reactive oxygen species by the mitochondrial voltage-dependent anion channel. Cancer Biol Ther 5:1523–1529

Sperry J, Bachu P, Brimble MA (2008) Pyranonaphthoquinones—isolation, biological activity and synthesis. Nat Prod Rep 25:376–400

Sperry J, Yuen TY, Brimble MA (2009) Enantioselective synthesis of the 3C-protease inhibitor (−)-thysanone by a Stauton-Weinreb annulation strategy. Synthesis 2009:2561–2569

Stipanovic RD, Zhang JX, Bruton BD, Wheeler MH (2004) Isolation and identification of hexaketides from a pigmented *Monosporascus cannonball*us isolate. J Agric Food Chem 52:4109–4112

Suetrong S, Schoch CL, Spatafora JW, Kohlmeyer J, Volkmann-Kohlmeyer B, Sakayaroj J, Phongpaichit S, Tanaka K, Hirayama K, Jones EBG (2009) Molecular systematics of the marine Dothideomycetes. Stud Mycol 64:155–173

Tatum JH, Baker RA, Berry RE (1987) Naphthofurans produced by *Fusarium oxysporum* isolated from citrus. Phytochemistry 26: 2499–2500

Thomson RH (1971) Naturally occurring quinones, 2nd edn. Academic Press, New York

Van Wagoner RM, Mantle PG, Wright JLC (2008) Biosynthesis of scorpinone, a 2-azaanthraquinone from *Amorosia littoralis*, a fungus from marine sediment. J Nat Prod 71:426–430

Whyte AC, Gloer KB, Gloer JB, Koster B, Malloch D (1997) New antifungal metabolites from the coprophilous fungus *Cercophora sordarioides*. Can J Chem 75:768–772

Yamamoto Y, Kinoshita Y, Thor GR, Hasumi M, Kinoshita K, Koyama K, Takahashi K, Yoshimura I (2002) Isofuranonaphthoquinone derivatives from cultures of the lichen *Arthonia cinnabarina* (DC.) Wallr. Phytochemistry 60:741–745