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Minutellins A – D, azaphilones from the stromata of *Annulohypoxylon minutellum* (Xylariaceae)

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ABSTRACT

During the course of our screening for new metabolites with chemotaxonomic importance from stromata of fungi from the family Xylariaceae, we characterized several interesting metabolites in the fungus *Annulohypoxylon minutellum*. Extraction of the fruiting bodies and purification by preparative HPLC resulted in the isolation of five metabolites. The main compound was identified as the known metabolite hinnulin A (**5**), while four minor compounds were found to represent previously undescribed azaphilones, named minutellins A – D (**1**–**4**). Their planar structures were elucidated using NMR and HRESIMS data; absolute stereochemistry was assigned by CD data and Mosher's method. Compounds **1, 3** and **5** showed cytotoxic effects against murine and human cells. As the production of **1**–**5** is restricted to a group of closely related *Annulohypoxylon* species, they serve well as chemotaxonomic marker.

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1. Introduction

The Xylariaceae is one of the most diverse taxonomic groups among the Ascomycota and species of this fungal family have been frequently isolated from plants as endophytes or pathogens (Karwehl and Stadler, 2016). The life cycle of some of these xylariaceous endophytes was shown to be mediated by insects (Pažoutová et al., 2013). Nevertheless, not much is known about the ecology of many of their species. Xylariaceae have become a common target by natural product researchers over the last decades and Whalley and Edwards (1995) have already demonstrated that their secondary metabolites are often of chemotaxonomic significance. In particular, the genera *Daldinia* (Stadler et al., 2014), *Hypoxylon* (Kuhnert et al., 2014b) and their allies have been found to produce an extremely high diversity of secondary metabolites. These are also abundantly present as pigments in their ascostromata (fruiting bodies). In the course of our chemotaxonomic studies, we are focusing on the isolation of new stromatal pigments from the under investigated tropical and subtropical representatives of *Hypoxylon sensu stricto* and its "sister genus", *Annulohypoxylon*. Consequently, we reported a variety of novel structures comprising tetramic acids such as the hypoxyvermelhotins (Kuhnert et al., 2014a), azaphilones including the cohaerins G-K (Surup et al., 2013), hydroxylated mitorubrin derivatives (Sir et al., 2015) or the lenormandins (Kuhnert et al., 2015), benzo[*j*]fluoranthenes (Sudarman et al., 2016) and prenylated indole derivatives such as the truncaquinones (Surup et al., 2016). One of the hitherto less studied species is *Annulohypoxylon minutellum*, formerly known under the synonym *Hypoxylon cohaerens* var. *microsporum*. This species is known from various tropical and subtropical countries and was previously identified by Quang et al. (2005a), (2005b) to produce interesting metabolites that were tentatively identified as cohaerin type azaphilones in the course of a chemotaxonomic survey of the section *Annulata* of *Hypoxylon* (now known as the genus *Annulohypoxylon*; cf. Kuhnert et al., 2016). However, lack of material has previously prevented the isolation and identification of these metabolites. We found that a specimen collected in the Canary Islands contained sufficient material to attempt preparative work. Herein, we describe the isolation, structure elucidation and biological activity of four new azaphilones (**1**–**4**) obtained from stromatal extracts of *Annulohypoxylon minutellum*, together

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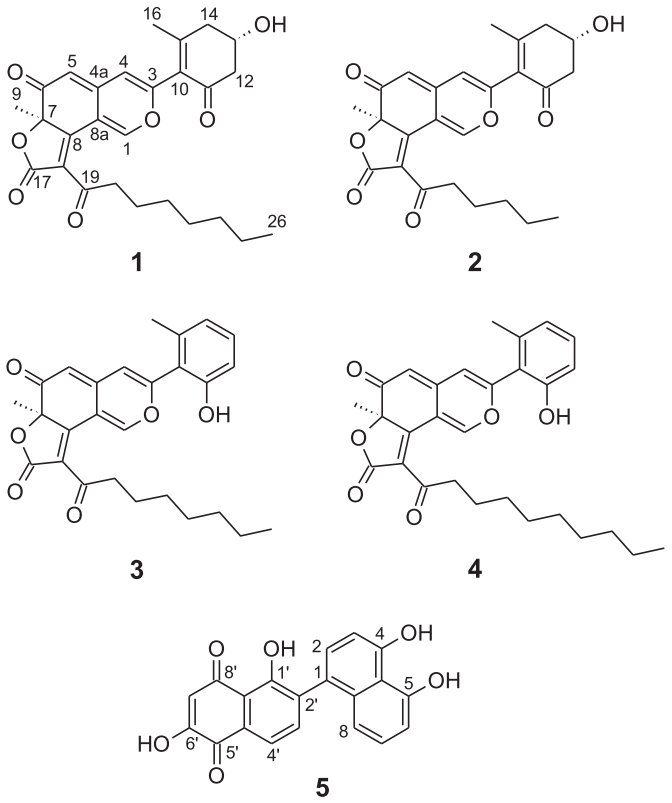

Fig. 1. Compounds isolated from the stromata of *Annulohypoxylon minutellum*: minutellins A-D (**1**–**4**), hinnulin A (**5**).

with the identification of hinnulin A (**5**) as its main pigment (Fig. 1).

2. Results and discussion

The analytical HPLC chromatograms based on UV and mass detection of extracts of *A. minutellum* showed the presence of two types of unidentified pigments (Fig. 2). Subsequently, metabolites **1**–**5** were isolated from the acetone extract of the stromata of *A. minutellum* using RP- and NP-HPLC.

Minutellin A (**1**) was isolated as a yellow oil, with the molecular formula $C_{27}H_{30}O_7$ determined by HRESIMS indicative of 13 units of unsaturation. The UV/Vis spectrum with absorption maxima of 227, 266 and 337 nm provided an early evidence for an azaphilone core structure (Quang et al., 2006). The proton and $^{1}H,^{13}C$ HSQC spectra showed signals of three methyls, three olefinic and one aliphatic methines along with eight methylenes. In addition, the carbon spectrum revealed the presence of three conjugated ketones, a single ester carbonyl, one oxygenated sp^3 hybridized quaternary and seven quaternary olefinic carbons. $^{1}H,^{13}C$ HMBC correlations from H1 to C3, C4a, C8, C8a; from H4 to C3, C4a, C5, C8a; from H5 to C4, C7, C8a and from $H_3$9 to C6, C7, C8 identified a highly conjugated azaphilone core (see Fig. 3). COSY correlations between $H_2$12/H13/$H_2$14/$H_3$16 and diverse HMBC correlations, especially from H13 to C11 & C15, $H_2$12 to C10/C11, and $H_3$16 to C10/C14/C15, assigned a 4-hydroxy-2-methyl-6-oxocyclohexyl moiety, attached to C3 because of HMBC correlations from H4, $H_3$16 and $H_2$14 to C10. Another entity was assigned as octan-1-one based on sequential COSY and TOCSY correlations from $H_2$20 to $H_3$26 and protons in between and HMBC correlations between $H_2$20/$H_2$21 and C19. The planar backbone of **1** with its angular five membered ring and the connection of the fatty acid side chain to C18 were concluded from the similarity of chemical shifts with cohaerin D (Quang et al., 2006) and multiformin B (Quang et al., 2005b). The stereochemistry of C7 was addressed by CD spectroscopy. The CD spectrum of **1** shows a negative Cotton effect at 417 nm and positive effects at 338 and 213 nm, indicating a 7*R* stereochemistry alike to the structurally closely related cohaerins (Quang et al., 2006; Surup et al., 2013). The absolute stereochemistry of C13 was assigned by Mosher's method. Because $\Delta\delta^{SR}$ values of α-methoxy-α-(trifluoro-methyl-) phenylacetic acid (MTPA) esters were negative for $H_3$16 and $H_2$14, respectively positive for H_a12 (see Fig. 4), a 13*S* configuration was deduced. In summary, **1** was assigned as (6a*R*)-3-[(4*S*)-4-hydroxy-2-methyl-6-oxocyclohex-1-en-1-yl]-6a-methyl-9-octanoyl-6*H*-furo[2,3-*h*]isochromene-6,8(6a*H*)-dione, amounting to the 27-desmethyl derivative of cohaerin D (Quang et al., 2006).

Minutellin B (**2**) had the molecular formula $C_{25}H_{26}O_7$, which indicated the formal loss of a C_2H_4 fragment compared to minutellin A (**1**). The proton and carbon spectra of **2** were very similar to **1**, with the key differences of the absence of two methylene signals in the saturated fatty acid side chain. Again, positive/negative Cotton effects at 352/421 nm in the CD spectrum indicated a 7*R* configuration of **2**. Consequently, **2** was assigned as (6a*R*)-9-hexanoyl-3-[(4*S*)-4-hydroxy-2-methyl-6-oxocyclohex-1-en-1-yl]-6a-methyl-6*H*-furo[2,3-*h*]isochromene-6,8(6a*H*)-dione.

For minutellin C (**3**), a molecular formula of $C_{27}H_{28}O_6$ was identified by HRESIMS data, indicating a formal loss of water in comparison to **1**. The proton and carbon spectra were very similar to those of **1** (see Tables 1 and 2), except for all signals of the substituent connected to C3 of the azaphilone core. An aromatic system was suggested by the chemical shifts of protons H12, H13 and H14, respectively carbons C10-C15 and coincidental disappearance of aliphatic signals in the moiety. Based on COSY cross-peaks between H12/H13/H14 and HMBC correlations from $H_3$16 to C10/C11/C12, the moiety was identified as 3-methylphenol. The configuration of the sole stereocenter at C7 was assigned as 7*R* based on its CD spectrum. Therefore, minutellin C (**3**) is the 27-desmethylderivative of cohaerin E, (6a*R*)-3-(2-hydroxy-6-methylphenyl)-6a-methyl-9-octanoyl-6*H*-furo[2,3-*h*]isochromene-6,8(6a*H*)-dione.

The yellow pigment minutellin D (**4**) had a molecular formula of $C_{29}H_{32}O_6$, a C_2H_4 unit heavier than **3**. The proton and carbon NMR data of **4** were nearly identical to that of **3**, with the exception of two additional methylene signals. As for **1**–**3**, the 7*R* configuration was indicated by CD data. Therefore minutellin D (**4**) was assigned as (6a*R*)-9-decanoyl-3-(2-hydroxy-6-methylphenyl)-6a-methyl-6*H*-furo[2,3-*h*]isochromene-6,8(6a*H*)-dione.

The molecular formula of the main pigment hinnulin A (**5**) was identified as $C_{20}H_{12}O_6$ by HRESIMS data, implying 15 degrees of unsaturation. In the carbon spectrum all resonances were observed above δ_C 109 ppm, indicative of a fully unsaturated metabolite. Proton and HSQC spectra accounted for eight olefinic/aromatic methines and one exchangeable proton. By COSY and HMBC NMR correlations, **1** was identified as hinnulin A. It is worth to note the deviation of carbon shifts between our data and those published by Schlingmann et al. (2011): Whereas Schlingmann et al. reported δ_C 171.4 for C6′, we measured δ_C 160.6, a difference of 10.8 ppm. A different protonation level of C6′–OH caused by a different isolation procedure was assessed as the most likely reason. Therefore, ^{1}H and ^{13}C NMR spectra of **5** were measured with an excess of CD_3COOD and NaOD, respectively. The excess of acid yielded in chemical shifts like obtained by us earlier, whereas data obtained with the excess of base resembled the ones of Schlingmann et al. (2011). This observation represents a scenario where a metabolite identification using carbon chemical shifts is complicated because the compound has the potential to act as weak acids/bases.

Compounds **1**, **3** and **5** exhibited moderate to weak cytotoxic

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