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

Eric Kuhnert^{1,2}, Frank Surup^{1,2}, Sandra Halecker^{1,2}, Marc Stadler^{1,2,*}

¹ *Helmholtz Centre for Infection Research GmbH (HZI), Department Microbial Drugs,
Inhoffenstraße 7, 38124 Braunschweig, Germany*

² *German Centre for Infection Research Association (DZIF), partner site Hannover-
Braunschweig, Inhoffenstraße 7, 38124 Braunschweig, Germany*

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.

Key words: Ascomycota, binaphthyls, biodiversity, chemotaxonomy, dereplication, polyketides, Xylariales

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 & 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 & 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 a extremely high diversity of secondary metabolites. These are In particular which 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 underinvestigated 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., 2014), 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. (2005) 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 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 $^1H,^{13}C$ 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. $^1H,^{13}C$ HMBC correlations from H1 to C3, C4a, C8, C8a; from H4 to C3, C4a, C5, C8a; from H5 to C4, C7, C8a and from H₃9 to C6, C7, C8 identified a highly conjugated azaphilone core (see Fig. 3). COSY correlations between H₂12/H13/H₂14/H₃16 and diverse HMBC correlations, especially from H13 to C11 &

C15, H₂12 to C10/C11, and H₃16 to C10/C14/C15, assigned a 4-hydroxy-2-methyl-6-oxocyclohexyl moiety, attached to C3 because of HMBC correlations from H4, H₃16 and H₂14 to C10. Another entity was assigned as octan-1-one based on sequential COSY and TOCSY correlations from H₂20 to H₃26 and protons in between and HMBC correlations between H₂20/H₂21 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 $\Delta\delta^{\text{SR}}$ values of α -methoxy- α -(trifluoro-methyl)-phenylacetic acid (MTPA) esters were negative for H₃16 and H₂14, respectively positive for H_a12 (see Fig. 4), a 13*S* configuration was deduced. In summary, **1** was assigned as (6*aR*)-3-[(4*S*)-4-hydroxy-2-methyl-6-oxocyclohex-1-en-1-yl]-6*a*-methyl-9-octanoyl-6*H*-furo[2,3-*h*]isochromene-6,8(6*aH*)-dione, amounting to the 27-desmethyl derivative of cohaerin D (Quang et. al 2006).

Minutellin B (**2**) had the molecular formula C₂₅H₂₆O₇, which indicated the formal loss of a C₂H₄ 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 (6*aR*)-9-hexanoyl-3-[(4*S*)-4-hydroxy-2-methyl-6-oxocyclohex-1-en-1-yl]-6*a*-methyl-6*H*-furo[2,3-*h*]isochromene-6,8(6*aH*)-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 & 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 crosspeaks between H12/H13/H14 and HMBC correlations from H₃16 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-desmethyl derivative of cohaerin E, (6*aR*)-3-(2-hydroxy-6-methylphenyl)-6*a*-methyl-9-octanoyl-6*H*-furo[2,3-*h*]isochromene-6,8(6*aH*)-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 (6*aR*)-9-decanoyl-3-(2-hydroxy-6-methylphenyl)-6*a*-methyl-6*H*-furo[2,3-*h*]isochromene-6,8(6*aH*)-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, ^1H 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 activity against the murine fibroblast cell line L929 and the human cervix carcinoma cell line KB3.1 in the range of 5.1 – 25 $\mu\text{g/mL}$. Weak antimicrobial activity against *Micrococcus luteus* was observed for **1**, **3**, **4** and **5** (16.7 – 66.7 $\mu\text{g/mL}$). In addition, **3** and **5** showed weak antifungal effects against the filamentous fungus *Mucor hiemalis* (66.7 $\mu\text{g/mL}$). For details on the MIC and tested organisms see the supplemental information.

Hinnulin A (**5**) was originally obtained from cultures of *Nodulisporium hinnuleum* and belongs to a class of binaphthyl-derived compounds (Schlingmann et al. 2011) that are frequently encountered in the Hypoxyloideae. As *Nodulisporium* represents the typical asexual morph of *Annulohypoxylon* it is likely that the original producer of **5** is closely related to *A. minutellum*. However, the lack of phylogenetic data on the producer strain prevents a taxonomic assessment. Binaphthyls produced by xylariaceous fungi can be separated into two groups depending on the arrangement of the naphthyls. Compound **5** belongs to the 1,2'-binaphthyl group and similar compounds have been reported from various sources such as hypoxylone from the stromata of members of the *H. polyporus* complex and *H. lividipigmentum* (Kuhnert et al., 2014b, Sir et al., 2016), urceolone from *A. urceolatum* (Bitzer et al. 2007), nodulisporin A from a culture of an unidentified *Nodulisporium* species (Dai et al., 2006), and daldionin from a culture of an

endophytic *Daldinia eschscholtzii* (Barnes et al., 2016). 1,1'-binaphthyls constitute the second group and typical representatives are BNT (binaphthalene tetrol), daldinones, hypoxylonols, and truncatones (Quang et al., 2002, Sudarman et al., 2016). It has been proposed that all binaphthyl-related compounds from fungi are derived from the 1,8-dihydroxynaphthalene (DHN) polyketide pathway, which is crucial for fungal melanin biosynthesis (Stadler & Fournier 2006, Barnes et al., 2016). The frequency of these compounds in hypoxylloid taxa suggests that the underlying biosynthetic pathway is well conserved within the subfamily. When screening stromatal extracts of more than 1000 Xylariaceae specimens, we discovered that the occurrence of hinnulin A in stromata is restricted to the closely related species *A. minutellum*, *A. ilanense*, and *A. purpureopigmentum* (Fig. 2) proving the significance of this compound as chemotaxonomic marker. The cytotoxicity and antimicrobial activity of **5** was demonstrated for the first time. Of the known 1,2'-binaphthyls only daldionin has been screened for biological activity and was shown to possess antiproliferative activity against various cell lines (Barnes et al., 2016) as well as broad spectrum activity against gram-positive bacteria. It has been proposed that the activity of naphthalenediol containing compounds might be linked to their high affinity for reactive oxygen species (ROS) resulting into an activation of the molecule by oxidation (Schlingmann et al., 2011).

Minutellins A – D (**1 – 4**) are azaphilones closely related to the cohaerins known from *A. cohaerens* (Quang et al., 2005a; 2006, Surup et al.; 2013) and the multiformins from *A. multiforme* (Quang et al.; 2005b), but lack the methyl group branching at the fatty acid side chain. The structural similarity is well reflected by the close taxonomic position of *A. cohaerens*, *A. multiforme*, and *A. minutellum* with the latter being originally described as *A. cohaerens* var. *microspora* (Ju & Rogers 1996). Additional members of the genus are known to produce

azaphilones as well (Quang et al., 2005a); we found minutellins to be present in stromatal crude extracts of *A. gombakense* (with **2** being the major constituent), *A. ilanense* (with **1 – 4** as minor constituents), and *A. michelianum* (with **4** being a major constituent) but lacking in *A. cohaerens* and *A. multiforme* (Fig. 2). Similar to hinnulin A (**5**), minutellins only occur in a restricted range of closely related taxa and hence serve well as chemotaxonomic markers to distinguish *Annulohypoxyton* species. However, structurally similar longirostrerones A-D have been isolated from the unrelated *Chaetomium longirostre* (Panthama et al., 2011). In general, azaphilones have been reported from various ascomycetes such as *Aspergillus*, *Penicillium*, *Chaetomium*, *Talaromyces*, *Pestalotiopsis*, *Phomopsis*, *Emericella*, *Monascus* and *Epicoccum*, as well as a few basidiomycetes including species of *Cyathus* and *Nidularia* (Gao et al., 2013). Moreover, they are known to exhibit a broad range of activities such as anti-HIV, anticancer, anti-inflammatory, antioxidative, antimicrobial and antihypertense effects (Gao et al., 2013). In our biological screening panel compounds **1** and **3** displayed activity against human and murine cell lines and weak antibacterial effects (in particular *B. subtilis*) were found for all azaphilones. This observation is in accordance with previously reported activities of structural related compounds such as the cohaerins and the longirostrerones (Surup et al., 2013, Panthama et al., 2011).

3. Conclusions

We isolated four new azaphilones (**1 – 4**), named minutellins A – D, and the binaphthyl hinnulin A (**5**) from the stromata of the fungus *Annulohypoxyton minutellum*. Compounds **1**, **3**, and **5** exhibited moderate to weak cytotoxic activity against human and murine cell lines. These secondary metabolites were produced by a close range of *Annulohypoxyton* species; they expand

the range of known pigments from the fruiting bodies of Xylariaceae and are valuable as chemotaxonomic markers.

4. Experimental section

4.1. General

Optical rotations were determined with a Perkin-Elmer 241 spectrometer and UV spectra were recorded with a Shimadzu UV-Vis spectrophotometer UV-2450. NMR spectra were recorded using a Bruker Avance III 700 spectrometer with a 5 mm TCI cryoprobe (^1H 700 MHz, ^{13}C 175 MHz) and Avance III 500 (^1H 500 MHz, ^{13}C 125 MHz) spectrometers. HRESI-MS spectra were obtained as described by Halecker et al. (2014). Isolation of pure compounds was achieved if not indicated otherwise using a preparative HPLC (Gilson, Middleton, USA) equipped with a GX-271 Liquid Handler, a 172 DAD, a 305 and 306 pump (with 50SC Piston Pump Head). As stationary phase a VP Nucleodur C18 ec column (125 × 40 mm, 7 μm ; or 250 × 21 mm, 5 μm ; Macherey-Nagel) was used. The mobile phase was composed of acid-free deionised water (Milli-Q, Millipore, Schwalbach, Germany; solvent A1) and acid-free acetonitrile (ACN; solvent B1). Flow rate was set to 15 or 30 mL/min, respectively.

4.2. Fungal material

Stromata (fruiting bodies) of *Annulohypoxylon minutellum* were collected in 2004 from the Canary Island La Palma (Spain) by M. Stadler. The species was unambiguously determined by morphological characterization and a voucher specimen has been deposited with the Staatliches Museum für Naturkunde (Germany, Karlsruhe; specimen designation KR-M-0002762).

4.3. Extraction and isolation

The stromata were carefully detached from the woody substrate (1.1 g) and extracted three times in each case with 60 mL acetone in an ultrasonic bath for 30 min at 40 °C. The extracts were filtered, combined and evaporated to yield 134 mg of crude extract. The latter was separated by preparative HPLC using a linear gradient of solvent B from 30 % to 100 % in 35 min followed by isocratic condition for 10 min. Fractions were collected every 20 seconds and combined according to the observed peaks. The main fraction with a retention time (RT) of 18.0-19.0 min (6 mg) contained a mixture of **1** and **3** and was further separated by normal phase HPLC applying the following conditions: mobile phase – dichloromethane (solvent A2), acetone (solvent B2); stationary phase – VP unmodified Nucleodur (250 × 21 mm, 5 µm; Macherey-Nagel); solvent B from 0 % to 20 % in 2 min, isocratic conditions for 20 min; flow rate 15 mL/min; detection at 220 nm and 340 nm. **1** was obtained in pure amounts at a RT of 7.0-8.0 min (2.7 mg) and **3** at a RT of 8.0-9.0 min (0.3 mg). Compound **2** resulted from the first fractionation at a RT of 23.0 min (2.2 mg). Peaks at a RT between 30.0 and 35.0 min were combined (7.4 mg) and again separated by reversed phase HPLC using a linear gradient from 75 % to 100 % solvent B1 in 30 min to yield **4** at a RT of 13.0-14.0 min (0.5 mg) and **5** at a RT of 19.0-20.0 min (0.5 mg).

4.4. Spectroscopic and spectrometric data

4.4.1. Minutellin A (**1**)

Yellow oil; $[\alpha]_D^{25} + 11$ (c 0.2, acetone); CD (CH₃CN) $\lambda_{\max}$ (δ_ϵ): 213 nm (+11.9), 277 nm (-13.5), 338 nm (14.1), 417 nm (-8.3); UV (CH₃CN) $\lambda_{\max}$ (log ϵ): 227 nm (3.95), 266 nm (3.89), 337 nm (3.85), 421 (sh); for ¹H NMR data, see table 1; for ¹³C NMR data, see table 2; ESIMS *m/z* 467.20

[M+H]⁺, 465.11 [M-H]⁻; HRESIMS *m/z* 467.2064 [M+H]⁺ (calcd. for C₂₇H₃₁O₇, 467.2064); R_t = 12.1 min.

4.4.2. Minutellin B (2)

Yellow oil; CD (CH₃CN) λ_{max} (δ_ε): 212 nm (+13.5), 251 nm (-4.2), 276 (-7.6), 352 (+7.4), 421 nm (-5.0); UV (CH₃CN) λ_{max} (log ε): 229 nm (4.12), 267 nm (4.09), 339 nm (4.03), 407 (sh); for ¹H NMR data, see table 1; for ¹³C NMR data, see table 2; ESIMS *m/z* 439.21 [M+H]⁺, 437.10 [M-H]⁻; HRESIMS *m/z* 439.1758 [M+H]⁺ (calcd. for C₂₅H₂₇O₇, 439.1751); R_t = 10.6 min.

4.4.3. Minutellin C (3)

Yellow oil; CD (CH₃CN) λ_{max} (δ_ε): 212 nm (+12.1), 251 nm (-5.0), 283 (-5.6), 352 (+7.6), 423 nm (-4.2); UV (CH₃CN) λ_{max} (log ε): 264 nm (3.99), 278 nm (sh), 338 nm (4.03), 407 (sh); for ¹H NMR data, see table 1; for ¹³C NMR data, see table 2; ESIMS *m/z* 449.19 [M+H]⁺, 447.11 [M-H]⁻; HRESIMS *m/z* 449.1962 [M+H]⁺ (calcd. for C₂₇H₂₉O₆, 449.1959); R_t = 14.8 min.

4.4.4. Minutellin D (4)

Yellow oil; CD (CH₃CN) λ_{max} (δ_ε): 213 nm (+14.1), 252 nm (-5.8), 287 (-6.6), 352 (+8.9), 418 nm (-4.8); UV (CH₃CN) λ_{max} (log ε): 264 nm (4.06), 279 nm (sh), 338 nm (4.09), 407 (sh); for ¹H NMR data, see table 1; for ¹³C NMR data, see table 2; ESIMS *m/z* 477.22 [M+H]⁺, 475.16 [M-H]⁻; HRESIMS *m/z* 477.2274 [M+H]⁺ (calcd. for C₂₉H₃₃O₆, 477.2272); R_t = 16.4 min.

4.4.5. Hinnulin A (5)

Murrey red oil; UV (CH₃CN/H₂O) λ_{max}: 228, 296, 336, 432 nm; ¹H NMR (500 MHz, acetone-*d*₆): δ_H 12.83 (s, 1'OH), 7.74 (d, *J* = 7.6 Hz, H4'), 7.66 (d, *J* = 7.6 Hz, H3'), 7.27 (d, *J* = 7.8 Hz, H2),

7.24 (dd, $J = 8.4$ Hz, 7.6 Hz, H7), 7.01(br d, $J = 8.4$ Hz, H8), 6.90 (d, $J = 7.8$ Hz, H3), 6.84 (dd, $J = 7.6$ Hz, 0.8 Hz, H6), 6.28 (s H7'); ^{13}C NMR (125 MHz, acetone- d_6): δ_{C} 193.3 (qC, C8'), 181.7 (qC, C5'), 160.6 (qC, C6'), 160.4 (qC, C1'), 155.8 (qC, C4), 155.3 (qC, C5), 139.4 (qC, C2'), 138.2 (CH, C3'), 135.9 (qC, C8a), 130.7 (qC, C4a'), 129.6 (CH, C2), 128.0 (CH, C7), 126.8 (qC, C1), 119.6 (CH, C4'), 118.7 (CH, C8), 115.7 (qC, C4a), 115.3 (qC, C8a'), 111.4 (CH, C7'), 109.9 (CH, C6), 109.3 (CH, C3); ESIMS m/z 349.08 $[\text{M}+\text{H}]^+$, 346.97 $[\text{M}-\text{H}]^-$; ESIMS m/z 349.08 $[\text{M}+\text{H}]^+$, 346.97 $[\text{M}-\text{H}]^-$; HRESIMS m/z 349.0709 $[\text{M}+\text{H}]^+$ (calcd. for $\text{C}_{20}\text{H}_{13}\text{O}_6$, 349.0707); $R_t = 10.2$ min.

4.5. Mosher's analysis of minutellin A (**1**) with MTPA chlorides

For the preparation of the (*S*)-MTPA ester 0.2 mg of **1** was dissolved in 600 μL of pyridine- d_5 , and 5 μL of (*R*)-MTPA chloride was added. The mixture was incubated at 25 $^{\circ}\text{C}$ for 15 min before measurement of ^1H , COSY, TOCSY and HSQC NMR spectra: ^1H NMR (700 MHz, pyridine- d_5): similar to **1**, but δ_{H} 9.12 (s, H1), 6.28 (s, H4), 5.87 (m, H13), 5.52 (s, H5), 3.35 (m, H_a12), 3.19 (dd, $J = 16.5$ Hz, 3.6 Hz, H_b12), 3.10 (m, H_a14), 2.85 (dd, $J = 19.2$ Hz, 4.5 Hz, H_b14), 1.93 (s, H₃16). The (*R*)-MTPA ester was prepared in the same manner by the addition of 5 μL of (*S*)-MTPA chloride: ^1H NMR (700 MHz, pyridine- d_5): similar to **1**, but δ_{H} 9.14 (s, H1), 6.40 (s, H4), 5.87 (m, H13), 5.53 (s, H5), 3.35 (m, H_a12), 3.17 (m, H_a14), 3.14 (m, H_b12), 2.95 (m, H_b12), 2.00 (s, H₃16).

4.6. Serial dilution and cytotoxicity assay

Minimum inhibitory concentrations (MIC) were determined in a serial dilution assay carried out in a similar manner as previously described (Surup et al., 2015) using various test organisms for antibacterial and antifungal activities. The *in vitro* cytotoxicity assay with the mouse fibroblast cell line L929 and the cervix carcinoma cell line KB3.1 (DSMZ no. ACC 158) was performed as previously reported (Surup et al., 2015).

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

Figure 2. Comparison of stromatal HPLC-UV profiles (200-600 nm) from various related *Annulohypoxylon* species. A – KR-M-0002762, B – holotype of *A. ilanense* (WSP 69730), C – KR-M-0002761, D – holotype of *A. gombakense* (WSP 69847), E – holotype of *A. purpureopigmentum* (WSP 71615). HA – hinnulin A (5), MA-MD – minutellins A-D (1-4).

Figure 3. Key $^1\text{H}, ^1\text{H}$ COSY (bold lines) and $^1\text{H}, ^1\text{H}$ HMBC (black arrows) correlations of minutellin A (**1**).

Figure 4. $\Delta\delta^{\text{SR}}$ values for MPTA esters of **1** diagnostic for the (*S*)-configuration of C13.

Table 1. ¹H NMR data (700MHz) for minutellins A – D (**1** – **4**).

position	1 ^a	2 ^b	3 ^b	4 ^b
1	8.77, br s	8.84, s	8.95, s	8.95, s
4	6.40, s	6.21, s	6.42, s	6.42, s
5	5.34, d (0.9)	5.35, s	5.40, s	5.41, s
9	1.67, s	1.73, s	1.76, s	1.77, s
12	2.73, dd (15.9,3.7)	2.83, m	6.76, d (8.2)	6.77, d (8.2)
	2.54, dd (15.9,7.3)	2.65, m		
13	4.33, m	4.42, br s	7.25, m	7.25, m
14	2.90, m	2.85, m	6.88, d (7.7)	6.88, d (7.7)
	2.64, m	2.65, m		
16	2.11, s	2.11, s	2.34, s	2.34, s
20	3.11, m	3.20, m	3.20, ddd(18.1,8.8,5.8)	3.21, ddd(18.1,8.9,5.9)
	2.90, m	2.83, m	2.85, ddd(18.1,8.8,5.8)	2.85, ddd(18.1,9.6,5.6)
21	1.61, m	1.61, m	1.62, m	1.62, m
22	1.31, m	1.31, m	1.31, m	
23	1.31, m	1.32, m	1.26, m	1.20-1.36, m
24	1.28, m	0.89, t (6.8)	1.25, m	
25	1.29, m		1.28, m	
26	0.87, t (7.0)		0.87, t (7.0)	1.26, m
27				1.29, m
28				0.88, t (7.0)

^a acetone-*d*₆, ^b chloroform-*d*

Table 2. ^{13}C NMR data (175MHz) for minutellins A – D (**1** – **4**).

position	1 ^a	2 ^b	3 ^b	4 ^b
1	154.4, CH	153.7, CH	154.0, CH	154.0, CH
3	154.9, C	152.9, C	154.8, C	154.8, C
4	113.8, CH	113.4, CH	113.0, CH	113.0, CH
4a	144.5, C	143.3, C	143.6, C	143.6, C
5	106.4, CH	106.3, CH	106.1, CH	106.1, CH
6	190.6, C	190.4, C	190.4, C	190.4, C
7	88.4, C	87.7, C	87.7, C	87.7, C
8	165.9, C	165.0, C	165.1, C	165.1, C
8a	112.0, C	111.2, C	111.3, C	111.3, C
9	26.3, CH ₃	26.3, CH ₃	26.4, CH ₃	26.4, CH ₃
10	130.7, C	130.1, C	118.7, C	118.7, C
11	194.8, C	193.4, C	153.8, C	153.8, C
12	47.0, CH ₂	46.1, CH ₂	113.7, CH	113.7, CH
13	65.8, CH	65.3, CH	131.7, CH	131.7, CH
14	41.9, CH ₂	41.0, CH ₂	123.1, CH	123.1, CH
15	162.6, C	161.1, C	139.1, C	139.1, C
16	23.0, CH ₃	23.1, CH ₃	20.1, CH ₃	20.1, CH ₃
17	169.3, C	168.0, C	168.1, C	168.1, C
18	124.6, C	123.8, C	123.9, C	123.9, C
19	197.6, C	197.2, C	197.3, C	197.3, C
20	41.4, CH ₂	42.1, CH ₂	42.2, CH ₂	42.2, CH ₂
21	24.2, CH ₂	23.1, CH ₂	23.4, CH ₂	23.4, CH ₂
22	30.0, CH ₂	31.2, CH ₂	29.0, CH ₂	29.0, CH ₂
23	30.0, CH ₂	22.4, CH ₂	29.1, CH ₂	29.7, 29.4, 29.2
24	32.5, CH ₂	13.9, CH ₃	31.7, CH ₂	3x CH ₂

25	23.3, CH ₂	22.6, CH ₂	
26	14.4, CH ₃	14.1, CH ₃	31.9, CH ₂
27			22.6, CH ₂
28			14.1, CH ₃

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^a acetone-*d*₆, ^b chloroform-*d*