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Botryane, noreudesmane and abietane terpenoids from the ascomycete *Hypoxylon rickii*

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KEYWORDS

Hypoxylon, Xylariaceae, abietane, botryane, eudesmane, terpenes, secondary metabolites, structure elucidation

ABSTRACT

In the course of our screening for new bioactive natural products, a culture of *Hypoxylon rickii*, a xylariaceous ascomycete collected from the Caribbean island Martinique, was identified as extraordinary prolific producer of secondary metabolites. Ten metabolites of terpenoid origin were isolated from submerged cultures of this species by preparative HPLC. Their structures were elucidated using spectral techniques including 2D NMR and HRESIMS. Three of the compounds were elucidated as new botryanes (**1** – **3**) along with three known ones, i.e. (3a*S*)-3a,5,5,8-tetramethyl-3,3a,4,5-tetrahydro-1*H*-cyclopenta[*de*]isochromen-1-one (**4**), (3a*S*,8*R*)-3a,5,5,8-tetramethyl-3,3a,4,5,7,8-hexahydro-1*H*-cyclopenta[*de*]isochromen-1-one (**5**) and botryenanol (**6**). Further three new sesquiterpenoids featured a 14-noreudesmane-type skeleton and were named hypoxylan A – C (**7** – **9**); the diterpenoid rickitin A (**10**) contains an abietane-type backbone. Compounds **1**, **2**, **3**, **7**, and **10** showed cytotoxic effects against murine cells.

Key words: Ascomycetes, biodiversity, endophytes, terpenoids, Xylariales

1. Introduction

The Xylariaceae (Ascomycota) is one of the largest fungal families, comprising more than 1300 accepted species (Stadler et al., 2013). Many members of the family are known to have an endophytic stage in their life cycle, but form fruiting bodies (stromata) only when their host is stressed or diseased (Stadler et al., 2014). Due to their endophytic lifestyle, many Xylariaceae are constantly being isolated from plant material samples and screened at random for their production of bioactive compounds. In particular, members of the genera *Daldinia* and *Hypoxylon* are often encountered among such endophytes, and the life cycle has only been elucidated very recently, using methods of molecular phylogeny (Bills et al., 2012, Pažoutová et al., 2013).

Cultures of such xylariaceous endophytes, or mycelia derived from ascospores of the genus *Hypoxylon* and related genera of the Xylariaceae have yielded various novel and interesting secondary metabolites in the past. Among those some compounds showed interesting bioactivity, such as the antiparasitic agents nodulisporic acid (Bills et al., 2012) and PF-1022A (Scherkenbeck et al., 2002), the topoisomerase 1 inhibitor hypoxyxylerone (Piettre et al., 2002), the immunomodulating dalescolols (Zhang et al., 2011), or the selective antifungal agents hypoxysordarin (Daferner et al., 1999) and sporothriolide (Surup et al., 2014). The fruiting bodies (stromata) of *Hypoxylon* and allied genera also proved to be a rather prolific source of new pigments and other secondary metabolites (Stadler and Fournier, 2006; Kuhnert et al., 2014a; Kuhnert et al., 2015; Surup et al., 2013). Interestingly, the major metabolites from the stromata of *Hypoxylon* are normally different from those that are encountered in the cultured mycelia and vice versa (Stadler, 2011).

Nevertheless, the majority of species in this extraordinarily diverse genus have never been tapped by secondary metabolite screenings, and many species have not even been cultured yet.

During our secondary metabolite screening of extracts derived from cultures of Xylariaceae, we identified the ex-epitype strain of the species *Hypoxylon rickii* as an extraordinarily prolific producer of secondary metabolites. More than 100 different compounds were detected in the bioactive crude extracts of this strain during preliminary studies of its secondary metabolism using HPLC-DAD/MS profiling. The fungus was therefore subjected to scale-up of fermentation in 70 litre scale, to conduct a case study on the chemical diversity of the produced metabolites. In this paper, we report the isolation, structure elucidation and biological activities of ten terpenoids (Figure 1), seven of which constitute new natural products.

2. Results and Discussion

Metabolites **1** – **10** were isolated from the acetone extract of the mycelium of a single cultivation of *H. rickii* using RP-MPLC followed by RP-HPLC. The molecular formula of **1** was determined by HRESIMS as $C_{18}H_{22}O_2$. The proton NMR spectrum (Table 1) revealed the presence of five methyl and one methylene groups, and four olefinic methine and one aldehyde protons, whereas the ^{13}C NMR spectrum (Table 1) furthermore identified one ketone, four quaternary sp^2 and two quaternary sp^3 hybridized carbons. $^{1}H,^{13}C$ HMBC correlations (Figure 2A) established a botryane-type carbon skeleton. However, compared to dehydrobotrydienal, known from *Botryotinia squamosa* (Kimata et al., 1985), a genus that represents the sexual morph of the

hyphomycete genus *Botrytis*, the carbon backbone was extended at C-10 by a propenyl moiety. A large vicinal coupling constant ($J_{16,17}$ = 16.8 Hz) and a ROESY correlation between H-16/H_3-18 demonstrated a $\Delta^{16,17}$ *E* configuration.

HRESIMS of metabolite **2** provided the elemental composition $C_{15}H_{18}O_3$. The NMR spectra (Table 1) were similar to those of **1**. However, in the proton and HSQC spectra the signals for H-4, H-16, H-17, H_3-18 were missing, while in the carbon spectrum the ketone signals of C-10 and C-15 were replaced by a carboxyl and an oxygenated methylene. ^{1}H,^{13}C HMBC correlations from H_3-11 to C-1, C-2, and C-3 and from H-3 to C-1, C-5, and C-11 placed a phenol function at C-4. The lactone ring closure between carboxyl C-11 and methylene C-15 was deduced from the ^{1}H,^{13}C HMBC correlations and the deep field shifts of H_a-15 and H_b-15, establishing the structure of **2**.

Metabolite **3** possessed the same molecular formula $C_{15}H_{18}O_3$ as **2**; the proton and carbon NMR spectra (Table 1) of the isomer were very similar to that of **2**. The structural difference is the shift of the phenol function (δ_H 4.90) to C-3, which was indicated by ^{1}H,^{13}C HMBC correlations of H-4 to C-2, C-6, and C-8 and of 3-OH to C-2, C-3, and C-4.

(3a*S*)-3a,5,5,8-tetramethyl-3,3a,4,5-tetrahydro-1*H*-cyclopenta[*de*]isochromen-1-one (**4**), (3a*S*,8*R*)-3a,5,5,8-tetramethyl-3,3a,4,5,7,8-hexahydro-1*H*-cyclopenta[*de*]isochromen-1-one (**5**) and botryenanol (**6**) were identified by their NMR data in combination with their elemental compositions from HRESIMS data (Collado et al., 1996; Colmenares et al., 2002)[*].

[*] Trivial names 10-Oxodehydrodihydrobotrydial for **4** and 10-oxodihydrobotryr-1(9),4(5)-diendial for **5** were introduced by Collado et al. However, these names are confusing because the functional groups are not properly indicated. We did not attempt to change them for the sake of

Hypoxylan A (**7**) was obtained as colorless oil; its molecular formula $C_{14}H_{20}O_2$ was deduced from HRESIMS data. Proton and $^{1}H,^{13}C$ HSQC spectra (Table 2) contained signals of two methyl and four methylene groups (one of which was oxygenated) and two sp^2 methines in addition to two sp^3 hybridized methines. Furthermore, the ^{13}C NMR spectrum revealed the presence of four quaternary sp^2 carbons. $^{1}H,^{1}H$ COSY and TOCSY spectra established partial structures from the methylene group C-1 to C-14 and from C-12 to C-13. The structure of hypoxylan A (**7**) was therefore deduced from the $^{1}H,^{13}C$ HMBC correlations shown in Figure 2B.

Hypoxylan B (**8**) had the elemental composition $C_{15}H_{22}O_2$ established by HRESIMS, indicating an additional methyl group as compared to **7**. The NMR spectra of **8** (Table 2) were very similar to those of **7**, with the exception of an additional methoxy signal (δ_H 3.81, δ_C 55.6). $^{1}H,^{13}C$ HMBC correlations of the methoxy signal to C-7, C-8, and C-9 demonstrated that hypoxylan B (**8**) is the O-8 methyl derivative of **7**.

Hypoxylan C (**9**) had the molecular formula $C_{15}H_{20}O_2$ according to HRESIMS. The missing methyl H_3-13 and methine H-11 signals and the appearance of an exocyclic methylene group (δ_H 5.39, 5.22; δ_C 114.6) suggested that **9** is the 11,13-didehydro derivative of **8**, which was demonstrated by $^{1}H,^{13}C$ HMBC correlations from H_a-13 and H_b-13 to C-7, C-11, C-12. Because hypoxylan C (**9**) contains only one stereocentre, the negative optical rotation of **9** defines the 4*S* stereochemistry typical for eremophilanes comparable e.g. with calcalol (Naya et al., 1976).

Rickitin A (**10**) was isolated from the mycelial extract as a minor constituent. HRESIMS established the molecular formula $C_{20}H_{26}O_3$. Proton and $^{1}H,^{13}C$ HSQC NMR spectra revealed the

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consistency nor want to perpetuate the flawed nomenclature, so we prefer to use the formal IUPAC nomenclature instead.

presence of five methyl and three methylene groups, as well as three olefinic and one aliphatic methine carbons. The ^{13}C NMR spectrum contained signals for two additional ketones, three sp^3 hybridized quaternary carbons (one of which was oxygenated) and three sp^2 hybridized quaternary carbons. ^{1}H,^{1}H COSY and TOCSY NMR spectra indicated partial structures from C-1 to C-3 and the C-15/C-16/C-17 isopropyl moiety. The abietane skeleton was derived from the ^{1}H,^{13}C HMBC correlations (Figure 2C). The relative configuration was elucidated on the basis of ^{1}H,^{1}H ROESY correlations. The 5-OH signal, which was observed in DMSO-d_6, showed ROESY correlations to H_β-1, H_β-3 and CH_3-18. Key ROESY correlations on the α-face were those from CH_3-20 to H_α-1 and H_α-2. The observed data were consistent with a *trans* configuration between CH_3-20 and 5-OH. Rickitin A (**10**) has never been described as a natural product. However, it was formed as an intermediate in the total synthesis of (−)-taiwaniaquinone H (Jana et al., 2010). The observed positive optical rotation of the natural product and of synthetic **10** determine their absolute configuration as 5*R*,10*R*. Putative congeners of **10** (as judged from the MS data) have been observed in the crude extract and resulting HPLC fractions of *H. rickii*, but they have not yet been isolated in sufficient amounts for structure elucidation.

Compounds **1** – **6** belong to the class botryane terpenoids. The name was derived from the phytopathogenic ascomycete *Botrytis cinerea*, from which the first members of the compound class were isolated. Many derivatives are known to have phytotoxic effects and therefore are regarded as pathogenicity factors (Deighton et al., 2001). Meanwhile, similar metabolites were obtained from the genera *Hypocrea* (an ascospore isolate of a wood-degrading member of the Hypocreaceae; see Yuan et al., 2013), as well as from the xylariaceous genera *Geniculosporium* (an endophyte, see Krohn et al., 2005) and a strain of an ascospore-derived isolate of *Daldinia* (Qin et al., 2008). None of these genera is regarded to be pathogenic, even though the

Xylariaceae have sometimes been reported to cause disease symptoms in stressed and weakened host plants. Interestingly, Qin et al. (2008) even reported that they only obtained the botryanes after induction experiments during the fermentation of their *Daldinia* strain. Further studies should be conducted in order to evaluate the ecological role of botryane sesquiterpenoids in these fungi and their potential as pathogenicity factors during the life cycle of the saprotrophic and endophytic producer organisms.

The diterpenoid **10** is a phenanthrenequinone of the abietane scaffold similar to the tanshinones. Quinone abietanes often possess cytotoxic activity, triggered by inhibition of topoisomerase I or II (Fronza et al., 2012), which might also be the case for Rickitin A (**10**). Interestingly, abietanes were to the best or our knowledge hitherto only reported as plant metabolites (see overviews by Feliciano et al., 1993; Wang et al., 2006). Nevertheless, the core structure of abietanes is closely related to the pimaranes, which are commonly encountered in fungi. Pimaranes were even already isolated from various *Xylaria* species (Isaka et al., 2012; Isaka et al., 2014), indicating that the biosynthetic pathway for tricyclic terpenoids might be common within the family.

Hypoxylans A – C (**7 - 9**) have an unusual scaffold for eudesmane-type sesquiterpenes. This class of secondary metabolites is distributed throughout the plant family Asteraceae and these compounds show various biological activities (Wu et al. 2006). In addition, they were isolated as metabolites of various fungi including members of the Xylariaceae (Isaka et al., 2010; Isaka et al., 2014). Moreover, the 14-noreudesmane core structure was already reported from cultures of the basidiomycete *Resupinatus leightonii* (Sundin et al., 1993), and, according to the latter reference citing an unpublished thesis, also from a *Panellus* species, but it was so far not found in ascomycetes. According to our preliminary biological characterisation, using a standardised test panel, the terpenoids **1** – **10** were almost devoid of antimicrobial activity against various

gram-positive and gram-negative bacteria as well as several fungi (Table 3). Only **7** and **10** showed weak activity against *Staphylococcus aureus*, whereas **4** had a weak antifungal effect on *Rhodotorula glutinis*. In the cytotoxicity assay moderate activity could be observed for **1**, **2**, **3**, **7** and **10**.

Interestingly, the stromatal extracts of *H. rickii* (i.e, the epitype specimen described by Kuhnert et al., 2014b, as well as the type specimen in the New Your Botanical garden) had yielded virtually the same metabolites as the common European species *Hypoxylon fragiforme*. Mitorubrin type azaphilones and orsellinic acid (cf. Stadler and Fournier, 2006) were the major detectable metabolites in the fruiting bodies of this species. From these data, it could not have been predicted that the mycelial culture of *H. ricki* would show such an extraordinary diversity of terpenoids.

While little is known as yet about the metabolic diversity of Xylariaceae, a member of their parent order Xylariales, *Pestalotiopsis fici* (cf. Wang et al., 2015) has recently been subjected to extensive genomic and transcriptomic studies. The impressive number of 97 gene clusters that putatively encode for secondary metabolite biosynthesis has been made out in the genome of this endophytic fungus. These results, along with the findings of the current study, demonstrate that a plethora of novel beneficial metabolites remains to be discovered from the Xylariales and in particular the Xylariaceae. Even manifold terpenoids have recently been discovered from endophytic fungi (de Souza et al., 2011), and the genus *Hypoxylon*, of which most species are suspected to have an endophytic stage in their life cycle, may also prove to be an additional rich source of such compounds.

3. Conclusions

We isolated seven novel terpenoids belonging to three different structural families from a single cultivation of the xylariaceous fungus *Hypoxylon rickii.* Compounds **1** – **3** are new members of the botryane family, whereas **4** – **6** had been isolated previously. Hypoxyalins A – C (**7** – **9**) are 15-noreudesmanes that could be biosynthetically related to the eremophilanes. The diterpenoid rickitin (**10**) is the first fungal metabolite with an abietane backbone. These results point towards a hitherto unsuspected diversity of terpenoids in the Xylariaceae –a fungal family that has so far mainly been known for its diversity of polyketides and nitrogen-containing secondary metabolites.

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 with Bruker Avance III 700 spectrometer with a 5 mm TCI cryoprobe (^{1}H 700 MHz, ^{13}C 175 MHz), Bruker DMX-600 (^{1}H 600 MHz, ^{13}C 150 MHz) and Avance III 500 (^{1}H 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 with 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; Macherey-Nagel) was used. The mobile phase was composed of deionised water (Milli-Q, Millipore, Schwalbach, Germany) with 0.1 % acetic acid (solvent A; Roth) and acetonitrile (ACN) with 0.1 % acetic acid (solvent B). Flow rate was set to 15 ml/min.

4.2. Fungal material

Stromata (fruiting bodies) of *Hypoxylon rickii* MJF10324 were collected in 2010 from the Caribbean island Martinique by J. Fournier. The strain was designated as epitype of the species (Kuhnert et al. 2014b). The culture was derived by multispore isolation on YMG medium (1.0 % malt extract, 0.4 % glucose, 0.4 % yeast extract, pH 6.3) using the method outlined by Stadler et al. (2014) and has been deposited in public culture collections (MUCL 53309, CBS 129345).

4.3. Cultivation, extraction and isolation

4.3.1. Cultivation

To determine the optimal condition for secondary metabolite production *Hypoxylon rickii* was transferred to four different liquid media (YMG medium; Q6/2 medium: 1.0 % glycerol, 0.25 % glucose, 0.5 % cotton seed flour, pH 7.2; ZM/2 medium: 0.5 % molasses, 0.5 % oatmeal, 0.15 % glucose, 0.4 % sucrose, 0.4 % mannitol, 0.05 % edamine, 0.05 % ammonium sulphate, 0.15 % calcium carbonate, pH 7.2; HLX medium: 3.0 % sucrose, 1.0 % casamino acids, 0.1 % K_2HPO_4, 0.1 % yeast extract, 0.05 % $MgSO_4$ x $7H_2O$, 0.05 % KCl, 0.001 % $FeSO_4$ x $7H_2O$) in Erlenmeyer flasks (500 ml) filled each with 200 ml media. These media were chosen because previous studies (Bitzer et al., 2008; Stadler et al., 2003; including details on the suppliers of the ingredients) had revealed that, taken together, they are optimal for attaining complementary secondary metabolite profiles in filamentous ascomycetes. The submerged cultures were

incubated at 23 °C in the dark on a rotary shaker at 140 rpm. Fermentation was aborted after free glucose was consumed and the pH value had passed through a minimum.

4.3.2. Extraction and large scale fermentation

An aliquot (20 ml) of the culture broth was mixed with 20 ml ethyl acetate and extracted in an ultrasonic bath for 30 min. The organic phase was filtered over anhydrous sodium sulphate and evaporated. The remaining crude extract was dissolved in methanol and analyzed with an analytical HPLC equipped with a diode array detector (DAD) as described by Stadler et al. (2001). Quantity and quality of secondary metabolites was the highest in HLX and ZM/2 media. For up-scaling purposes, HLX medium was chosen. A seed culture of the strain with a total volume of 1 l was prepared in YMG medium and incubated for 7 days. A Biostat UE 100 bioreactor (B. Braun Melsungen AG, Germany) filled with 70 l HLX medium was inoculated with the seed culture under sterile conditions. To prevent foam formation in total 20 g of Tegosipon (Evonik, Essen, Germany) was added in portions of 2 to 10 g as necessary, using an automatic supply system. The pH value was maintained between 6.0 and 6.3 using potassium hydroxide (2 N) and sulphuric acid (1 M), respectively. The temperature was set at 26 °C. The oxygen saturation was kept above a minimum of 20 %, controlled by increasing the stirrer speed of the Rushton turbine by the built-in process control system. The initial stirrer speed was set to 50 rpm and reached a maximum speed of 400 rpm, aeration rate was set to 5 Sl/min and remained constant during fermentation. The culture was harvested after 7 days as sugars (sucrose, fructose) were depleted. Thereafter, the mycelium was separated from the culture fluid by vacuum filtration to yield a total amount of 3 kg wet biomass, which was later extracted with 9 l acetone in an ultrasonic bath for 1 h. The acetone extract was filtered and evaporated to yield an aqueous phase (3.5 l), which was further processed by extraction with 3x 1 litres of ethyl

acetate in a separating funnel. Subsequently the organic phases were combined, dried over anhydrous sodium sulphate and evaporated to yield 12 g of oily mycelial crude extract (ME) in total.

4.3.3. Isolation of the terpenoids

The ME containing all terpenoids was separated in three parts and each (4 g) dissolved in 12 ml methanol. Each part was later filtered through a RP solid phase cartridge (Strata-X 33 mm, Polymeric Reversed Phase; Phenomenex Aschaffenburg, Germany) and subjected to preparative RP MPLC [column 480 × 30 mm, ODS/AQ C18 (Kronlab) using the following conditions: mobile phase with solvent A [90 % deionised water (Milli-Q), 10 % methanol] and solvent B (methanol), linear gradient of solvent B from 10 to 100 % in 60 min, followed by isocratic conditions at 100 % solvent B for 20 min, flow rate of 30 ml/min, UV peak detection at 210 nm. A total number of 160 fractions per run were obtained, which were combined according to the main peaks to yield 15 main fractions. Each main fraction was evaporated and the crude extract analyzed by HPLC-DAD. One of the main fractions derived from the first run [retention time (RT): 56.0 – 57.0 min; 15 mg) contained pure compound **4**. Another fraction obtained in the first run (58.0 – 59.0 min, 16mg) was further fractionized by RP HPLC (linear gradient from 25% to 100% solvent B in 40 min) to obtain compounds **1** (0.75 mg) and **9** (0.73 mg). A further fraction derived from the first run (RT: 48.5 – 49.0 min; 34 mg) was again separated by preparative RP HPLC (linear gradient from 30 to 60 % solvent B in 30 min) to obtain compound **6** (7 mg) and [**10**] (3 mg). Compound [**3**] (4 mg) was purified from a main fraction of the second run (RT: 57.0 – 58.5 min; 59 mg) by preparative HPLC [linear gradient from 40 to 100 % in 25 min using 95 % ACN + 5 % deionised water supplemented with 0.015% formic acid (Roth) as solvent B]. Another fraction of the same run (RT: 43.0 – 45.5 min; 119 mg) was fractionized (linear gradient

from 30 to 70 % solvent B in 30 min) to yield compound **2** (4 mg). The adjacent fraction (RT: 45.5 – 49.0 min; 900 mg) was separated (linear gradient from 30 to 80 % solvent B in 25 min) to obtain the main compound **5** (370 mg) and compound **7** (50 mg). A main fraction of the third run (54.0 – 56.0 min, 65 mg) was separated by preparative RP HPLC (50% to 100% solvent B in 35 min) to obtain compound **8** (1.06 mg). All resulting fractions were analysed by HPLC-MS to follow the success of the purification.

4.4. Spectroscopic and spectrometric data

4.4.1 (1*S*)-7-[(2*E*)-but-2-enoyl]-1,3,3,6-tetramethyl-2,3-dihydro-1*H*-indene-1-carbaldehyde (**1**)

Colorless oil, $[\alpha]^{25}_{D}$ 0 (*c* 0.08, MeOH); UV (MeOH) λ_{max} (log ε) 282 nm (4.05); ^{1}H NMR, ^{13}C NMR data see Table 1; ESIMS *m/z* 293.04 $[M+Na]^+$; HRESIMS *m/z* 271.1696 $[M+H]^+$ (calcd for $C_{18}H_{23}O_2$ 271.1693); R_t = 12.4 min.

4.4.2 (3a*S*)-6-hydroxy-3a,5,5,8-tetramethyl-3,3a,4,5-tetrahydro-1*H*-cyclopenta[*de*]isochromen1-one (**2**)

Colorless oil, $[\alpha]^{25}_{D}$ +4 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 225 (sh), 271 nm (3.73); ^{1}H NMR, ^{13}C NMR data see Table 1; ESIMS *m/z* 247.00 $[M+H]^+$, 245.10 $[M-H]^-$; HRESIMS *m/z* 247.1330 $[M+H]^+$ (calcd for $C_{15}H_{19}O_3$ 247.1329); R_t = 9.8 min.

4.4.3 (3a*S*)-7-hydroxy-3a,5,5,8-tetramethyl-3,3a,4,5-tetrahydro-1*H*-cyclopenta[*de*]isochromen-1-one (**3**)

Colorless oil, $[\alpha]^{25}_{D}$ 0 (*c* 0.8, MeOH); UV (MeOH) λ_{max} (log ε) 249 nm (3.61), 325 nm (3.43); ^{1}H NMR data see Table 2; ^{13}C NMR data see Table 1; HRESIMS *m/z* 247.1331 $[M+H]^{+}$ (calcd for $C_{15}H_{19}O_3$ 247.1329); R_t = 9.7 min.

4.4.4 (3a*S*)-3a,5,5,8-tetramethyl-3,3a,4,5-tetrahydro-1*H*-cyclopenta[*de*]isochromen-1-one (**4**)

Colorless oil; ^{1}H NMR and ^{13}C NMR data were consistent with those previously reported (Colmenares et al. 2002); HRESIMS *m/z* 231.1392 $[M+H]^{+}$ (calcd for $C_{15}H_{19}O_2$ 231.1380); R_t = 12.2 min.

4.4.5 (3a*S*,8*R*)-3a,5,5,8-tetramethyl-3,3a,4,5,7,8-hexahydro-1*H*-cyclopenta[*de*]isochromen-1-one (**5**)

Colorless oil; ^{1}H NMR and ^{13}C NMR data were consistent with those previously reported (Colmenares et al. 2002); HRESIMS *m/z* 233.1537 $[M+H]^{+}$ (calcd for $C_{15}H_{21}O_2$ 233.1536); R_t = 11.6 min.

4.4.6 Botryenanol (**6**)

Colorless oil; ^{1}H NMR and ^{13}C NMR data were consistent with those previously reported (Collado et al. 1996); HRESIMS *m/z* 317.1722 $[M+H]^{+}$ (calcd for $C_{17}H_{26}O_4Na$ 317.1723); R_t = 9.5 min.

4.4.7 Hypoxylan A (**7**)

Colorless oil, $[\alpha]^{25}_{D}$ +42 (*c* 1.6, MeOH); UV (MeOH) λ_{max} (log ε) 223 nm (sh), 283 nm (3.37); ^{1}H NMR, ^{13}C NMR data see Table 2; ESIMS *m/z* 203.02 $[M+H-H_2O]^{+}$, 219.01 $[M-H]^{-}$; HRESIMS *m/z* 243.1351 $[M+Na]^{+}$ (calcd for $C_{14}H_{20}O_2Na$ 243.1356); R_t = 10.0 min.

4.4.8 Hypoxylan B (**8**)

Colorless oil, $[\alpha]^{25}_{D}$ +26 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 239 nm (3.61), 287 nm (3.19); ^{1}H NMR, ^{13}C NMR data see Table 2; ESIMS *m/z* 217.02 $[M+H-H_2O]^+$; HRESIMS *m/z* 235.1698 $[M+H]^+$ (calcd for $C_{15}H_{23}O_2$ 235.1693); R_t = 11.8 min.

4.4.9 Hypoxylan C (**9**)

Colorless oil, $[\alpha]^{25}_{D}$ 0 (*c* 0.07, MeOH); UV (MeOH) λ_{max} (log ε) 242 nm (sh), 287 nm (3.23); ^{1}H NMR, ^{13}C NMR data see Table 2; ESIMS *m/z* 215.00 $[M+H-H_2O]^+$, 233.02 $[M+H]^+$; HRESIMS *m/z* 233.1544 $[M+H]^+$ (calcd for $C_{15}H_{21}O_2$ 233.1536); R_t = 11.3 min.

4.4.9 Rickitin (**10**)

Colorless oil, $[\alpha]^{25}_{D}$ +112 (*c* 0.3, MeOH); UV (MeOH) λ_{max} (log ε) 282 nm (3.29); ^{1}H NMR (DMSO-d_6, 700 MHz) δ 7.74 (d, *J* = 2.2 Hz, H-14), 7.59 (dd, *J* = 8.2, 2.2 Hz, H-12), 7.41 (d, *J* = 8.2 Hz, H-11), 5.77 (brs, 5-OH), 2.96 (spt, *J* = 6.9 Hz, H-15), 2.17 (brd, *J* = 12.9 Hz, H_α-1), 2.06 (ddd, *J* = 13.2, 12.9, 4.1 Hz, H_β-1), 1.83 (m, H_α-2), 1.65 (m, H_β-3), 1.60 (m, H_β-2), 1.40 (s, H_3-18), 1.26 (s, H_3-20), 1.22 (d, *J* = 6.9 Hz, H_3-16, H_3-17), 1.09 (brd, J = 12.9 Hz, H_α-3), 0.99 (s, H_3-19); ^{13}C NMR δ (DMSO-d_6, 175 MHz) 195.0 (C-6), 186.3 (C-7), 152.2 (C-9), 146.5 (C-13), 133.9 (C-12), 130.8 (C-8), 125.1 (C-11), 124.1 (C-14), 82.8 (C-8), 46.5 (C-10), 36.7 (C-4), 36.3 (C-3), 32.7 (C-15), 30.8 (C-1), 28.8 (C-20), 28.2 (C-19), 23.6 (C-16, C-17), 23.4 (C-18), 17.8 (C-2); HRESIMS *m/z* 315.1947 $[M+H]^+$ (calcd for $C_{20}H_{27}O_3$ 315.1955); R_t = 13.9 min.

4.5. 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 (Halecker et al., 2014) 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 reported by Surup et al. (2013). The results of both assays are shown in Table 3.

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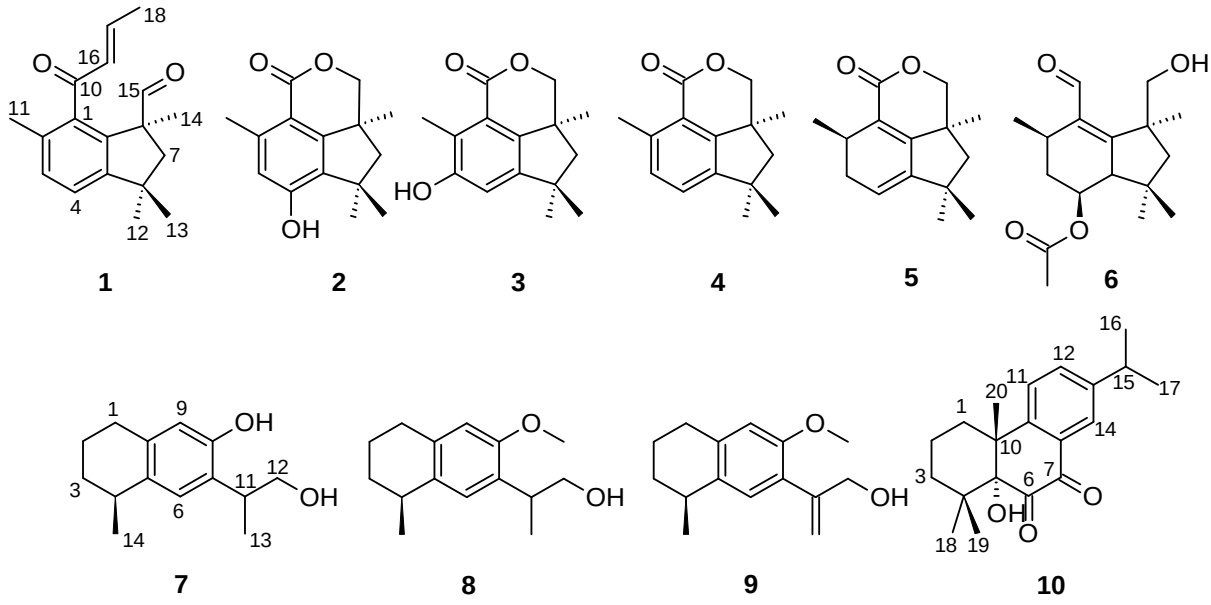

Figure 1. Terpenoids **1** – **10** isolated from a single cultivation of the fungus *Hypoxylon rickii*.

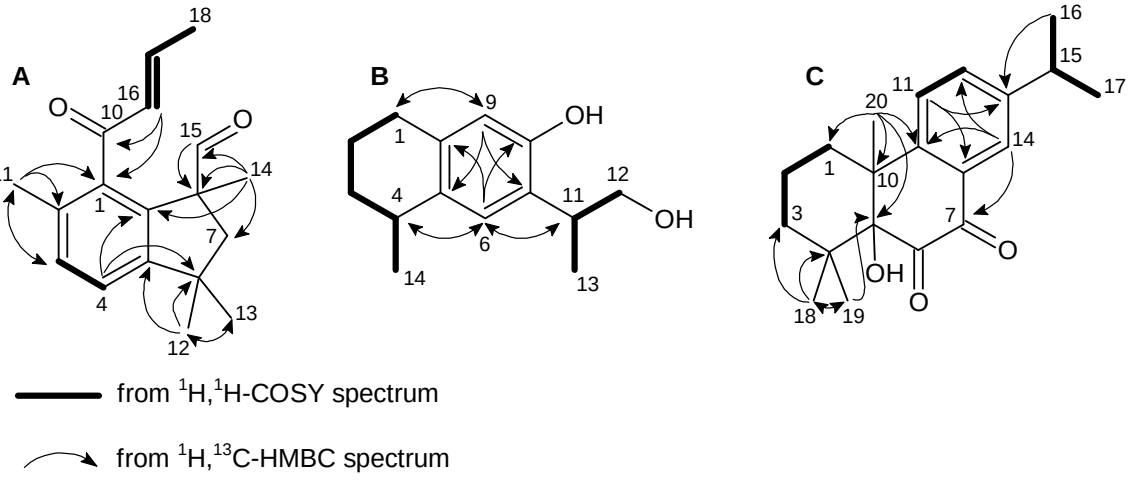

Figure 2. Selected COSY and HMBC correlations determining the carbon backbones of (A) **1**, (B) **7** and (C) **10**.

Table 1. NMR Spectroscopic Data of **1- 3** in Chloroform-*d*.

	1[a]		**2**[b]		**3**[b]	
1	132.2, C		112.5, C		120.4, C	
2	153.3, C		143.4, C		124.6, C	
3	131.9, CH	7.21, d (7.7)	118.7, CH	6.47, d (0.6)	155.0, C	
4	123.3, CH	7.09, d (7.7)	156.2, C		114.5, CH	6.84, s
5	151.8, C		130.4, C		147.8, C	
6	43.3, C		45.7, C		45.2, C	
7	49.9, CH_2	2.33, m 1.80, d (13.7)	52.4, CH_2	1.91, d (12.8) 1.85, d (12.8)	52.0, CH_2	1.96. d (13.2) 1.85, d (13.2)
8	59.1, C		41.7, C		40.7, C	
9	140.9, C		154.9, C		143.6, C	
10	198.6, C		164.1, C		164.1, C	
11	21.0, CH_3	2.34, m	20.5, CH_3	2.58, d (0.6)	11.6, CH_3	2.55, s
12	31.6, CH_3	1.36, s	29.7, CH_3	1.47, s	30.7, CH_3	1.45, s
13	30.9, CH_3	1.33, s	28.3, CH_3	1.53, s	30.6, CH_3	1.31, s
14	21.6, CH_3	1.49, s	25.0, CH_3	1.48, s	24.7, CH_3	1.49, s
15	201.5, CH	9.53, s	78.7, CH_2	4.32, d (10.1) 4.15, d (10.1)	79.7, CH_2	4.35, d (9.5) 4.10, d (9.5)
16	134.6, CH	6.23, d (16.8)				
17	140.8, CH	7.41, d (16.8)				
18	26.7, CH_3	2.34, m				

[a] ^{1}H at 700 MHz, ^{13}C at 175 MHz. [b] ^{1}H at 500 MHz, ^{13}C at 125 MHz.

Table 2. NMR Spectroscopic Data of **7- 9** in Chloroform-*d*.

	7[a]		**8**[b]		**9**[b]	
1	29.7, CH_2	2.67, m	30.3, CH_2	2.72, m	30.2, CH_2	2.76, m
2	20.5, CH_2	1.82, m	20.5, CH_2	1.84, m	20.4, CH_2	1.88, m
		1.67, m		1.69, m		1.72, m
3	31.8, CH_2	1.87, m	31.6, CH_2	1.89, m	31.6, CH_2	1.91, m
		1.49, m		1.51, m		1.53, m
4	31.9, CH	2.83, m	32.0, CH	2.83, dqd	31.7, CH	2.86, dqd
				(12.4,7.0,6.1)		(12.0,7.0,6.0)
5	134.3, C		134.3, C		134.5, C	
6	127.3, CH	6.92, s	127.2, CH	6.99, m	129.9, CH	7.05, s
7	128.2, C		129.3, C		127.0, C	
8	152.4, C		155.1, C		154.1, C	
9	116.9, CH	6.59, s	110.9, CH	6.55, s	110.9, CH	6.59, s
10	136.6, C		135.9, C		137.7, C	
11	36.8, CH	3.19, dqd	35.6, CH	3.34, dq	148.3, C	
		(7.8,7.3,3.7)		(7.3,7.2)		
12	69.7, CH_2	3.92, dd	68.2, CH_2	3.69, m	65.9, CH_2	4.43, br s
		(9.8,3.7)				
		3.72, dd				
		(9.8,7.8)				
13	15.8, CH_3	1.30, d (7.3)	16.7, CH_3	1.247, d (7.2)	114.6, CH_2	5.39, br s
						5.22, br s
14	23.1, CH_3	1.24, d (7.9)	23.1, CH_3	1.246, d (7.0)	22.9, CH_3	1.28, d (7.0)
8OMe			55.5, CH_3	3.78, s	55.6, CH_3	3.81, s

[a] ^{1}H at 500 MHz, ^{13}C at 125 MHz. [b] ^{1}H at 700 MHz, ^{13}C at 175 MHz.

Table 3. In vitro antibacterial, antifungal & cytotoxic activity of the terpenoids **1** – **10**, all dissolved in methanol, and three control drugs.

Test organisms	MIC[†] [µg/ml]										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Ref. [a,b,c]**
Gram-positive bacteria											
Bacillus subtilis DSM 10[‡]	-[§]	-	-	-	-	-	-	-	-	-	6.7[a]
Micrococcus luteus DSM 20030	-	-	-	-	-	-	-	-	-	-	4.2[a]
Mycobacterium sp. DSM 43270	-	-	-	-	-	-	-	-	-	-	8.3[a]
Staphylococcus aureus DSM 346	-	-	-	-	-	-	67.0	-	-	33.3	0.1 – 0.21[a]
Gram-negative bacteria											
Chromobacterium violaceum DSM 30191	-	-	-	-	-	-	-	-	-	-	1.0[a]
Escherichia coli DSM 1116	-	-	-	-	-	-	-	-	-	-	0.83 – 1.7[a]
Pseudomonas aeruginosa DSM 50071	-	-	-	-	-	-	-	-	-	-	1.0[b]
Yeasts											
Candida albicans DSM 1665	-	-	-	-	-	-	-	-	-	-	8.3[c]
Rhodotorula glutinis DSM 10134	-	-	-	33.3	-	-	-	-	-	-	1.0[c]
Schizosaccharomyces pombe DSM 70572	-	-	-	-	-	-	-	-	-	-	4.2 – 8.3[c]
Wickerhamomyces anomalus DSM 6766	-	-	-	-	-	-	-	-	-	-	8.3[c]
Filamentous fungi											
Mucor hiemalis DSM 2656	-	-	-	-	-	-	-	-	-	-	4.2[c]
Cell lines											
mouse fibroblast cell line L929	8.5	28.0	19.0	-	-	-	36.0	-	-	23.0	-
cervix carcinoma cell line KB3.1	n.t.[**]	26.0	n.t.	n.t.	n.t.	-	n.t.	n.t.	n.t.	18.0	-

[†] MIC: Minimum inhibitory concentration. The cell density was adjusted to 8 x 10^6 cells/ml.
[‡] DSMZ: German Collection of Microorganisms and Cell Cultures, Braunschweig
[§] -: no inhibition
[**] n.t.: not tested
[a] Oxytetracyclin hydrochloride (1 mg /ml), [b] Gentamycin (10 mg/ml), [c] Nystatin (1 mg/ml)