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Rickicaryophyllane A, a Caryophyllane from the Ascomyceteous Fungus *Hypoxylon rickii* and a 10-Norbotryane Congener

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Herein we report the isolation from *Hypoxylon rickii* of a new sesquiterpenoid (**1**) with a caryophyllane skeleton. The planar structure of **1** was elucidated by NMR and HRMS data as the 1,12-dihydro-1-hydroxyl derivative of caryophyllenol-I, for which we propose the name rickicaryophyllane A. Its relative stereochemistry was assigned with a series of 1D NOESY experiments, while the 1*R*,2*S*,5*R*,9*R* absolute configuration was demonstrated by Mosher`s analysis. Besides, we isolated 3-(hydroxymethyl)-1,1,3,5-tetramethyl-1,2,3,5,6,7-hexahydro-4*H*-inden-4-one (**2**) as a new 10-norbotryane derivative and the known metabolite orcacetophenone (**3**).

Keywords: Xylariaceae, Xylariales, Secondary metabolites, Structure elucidation, Sesquiterpenes.

Terpenoids constitute one of the main classes of natural products, which are formed from C5 units leading to their characteristic branched chain structure [1]. Fungi and particularly basidiomycetes are known to produce sesquiterpenoids as the most common category [2,3].

In the course of our screening program we are investigating fungal isolates for their potential to yield secondary metabolites. Because *Hypoxylon rickii* (family Xylariaceae) produced a variety of secondary metabolites during preliminary experiments in shake flasks, the strain was cultivated on a 70 L scale. From this single cultivation we obtained new terpenoids with botryane, 14-noreremophilane, abietane [4] and silphiperfolane type skeletons [5]. Moreover, we isolated a series of new terphenyls [6]. Herein we report the isolation and characterization of further caryophyllane and 10-norbotryane sesquiterpenoids.

The extract of the culture filtrate of this large scale 70 L fermentation [4] was partitioned between methanol and heptane. From the lipophilic part solved in heptane, compound **1** (see Figure 1) was isolated as a colorless oil, using RP-MPLC followed by RP-HPLC. Its molecular formula, $C_{15}H_{26}O_2$, was deduced from its $[M+Na]^+$ and $[M+H-H_2O]^+$ peaks in the HRESIMS. ^1H and $^1H,^{13}C$ HSQC spectra (see Table 1) revealed the presence of four methyls, four methylene, one olefinic and three aliphatic methines, one of them oxygenated. Carbon and $^1H,^{13}C$ HMBC spectra demonstrated one sp^2 hybridized and two sp^3 hybridized quaternary carbons, one of these oxygenated. $^1H,^1H$ COSY and TOCSY spectra established the spin systems H_2-3/H-2/H-5/H_2-6/H-7 and H-9/H_2-10/H_2-11 (see Figure 2). The nine membered ring structure was demonstrated as a result of $^1H,^{13}C$ HMBC correlations from H_3-12 to C-1/C-2/C-11 and H_3-15 to C-7/C-8/C-9. Because both geminal methyls H_3-13 and H_3-14 correlate to C-3 and C-5 in the HMBC spectrum, a cyclobutane ring moiety was deduced, establishing the planar caryophyllene backbone of **1** with its bicyclo[2.7.0]undecane skeleton. The elucidation of the relative configuration was seriously hampered because several signals are overlapping in the region of

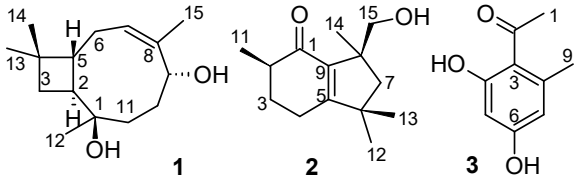

Figure 1: Structures of rickicaryophyllane A (**1**), 10-norbotryane derivative **2** and orcacetophenone (**3**).

δ_H 1.3 to 1.8 ppm in the $^1H,^1H$ NOESY spectrum. Therefore, we reexamined the compound in a series of 1D ROESY experiments (see Figure 3). Irradiation of 14- and 13-methyl protons caused an enhancement of H-5 and H-2, respectively, and, therefore, indicated that the cyclobutane ring is condensed in *trans* mode. The correlation between H_3-12 and H-2 is indicating an 1*R**,2*S**,5*R** relationship. The NOESY correlation between H-5/H-9 and H-7/H_3-15 pointed out a 9*R** and 7*Z* configuration, respectively. Metabolite **1** is the 1,12-dihydro-1-hydroxyl derivative of caryophyllenol-I [7]; therefore, we concluded an analogous absolute configuration based on the observed negative optical rotation. This was confirmed by a Mosher`s analysis with MTPA derivatives (see Figure 4). The distribution of negative $\Delta\delta^{SR}$ values for H-7/H_3-15 and positive values for H_2-10/ H_β-11/H_3-12 demonstrates the 1*R*,2*S*,5*R*,9*R* configuration [8].

Sesquiterpenoids with a caryophyllane backbone have commonly been isolated from plants [7,9]. Additionally, members of the family are well known from corals [10]; rumphellanol A, the 9-oxo derivative of **1**, has been isolated from the coral *Rumphella antipathies* [11]. Higher fungi are another source of caryophyllanes [12,13], so the isolation of **1** from *H. rickii* is not completely unanticipated.

Compound **2** was isolated as a colorless oil from the middle polar fraction solved in methanol, which was further partitioned between

Table 1: NMR data (^{1}H 700 MHz, ^{13}C 125 MHz) of rickicaryophyllane A (**1**) in CD_3OD.

Atom Number	δ_C, mult.	δ_H, mult.	COSY	HMBC	H_3-12[a]	H_3-13[a]	H_3-14[a]	H-9[a]
1	73.5, C							
2	42.9, CH	2.06, m	3a, 3b, 5	1,3,4,5,6,11	s	i	w	
3	34.4, CH_2	1.68, t (9.9)	2, 3b	1,2,4,5,13,14	s		s	
		1.42, dd (10.4,8.2)	2, 3a	1,2,4,5,13,14	i	s		
4	34.5, C							
5	45.1, CH	2.11, ddd (11.5,9.0,4.0)	2, 6	1,2,3,6,7	i	w	s	i
6	27.4, CH_2	2.03, m	5, 7	2,4,5,7,8		w		
		2.20, ddd (13.5,11.5,8.0)						s
7	125.7, CH	5.42, t (8.4)	6	5,6,9,15		s		
8	141.1, C							
9	74.6, CH	4.37, br d (7.0)	10a, 10b	7,8,10,11,15				
10	31.7, CH_2	1.50, m	9, 10b, 11a, 11b	1,8,9,11				
		1.73, m	9, 10a, 11a, 11b	1,8,9,11	i			
11	40.1, CH_2	1.71, m	10a, 10b, 11b	1,2,9,10,12	w			
		1.56, m	10a, 10b, 11a	1,2,9,10,12	i			s
12	26.4, CH_3	0.96, s		1,2,11				
13	24.8, CH_3	1.01, s		3,4,5,14				
14	30.5, CH_3	0.98, s		3,4,5,13				
15	19.8, CH_3	1.74, br s		7,8,9				

[a] Series of 1D ROESY experiments irradiating respective proton resulted in the appearance of denoted signals (s: strong, i: intermediate, w: weak).

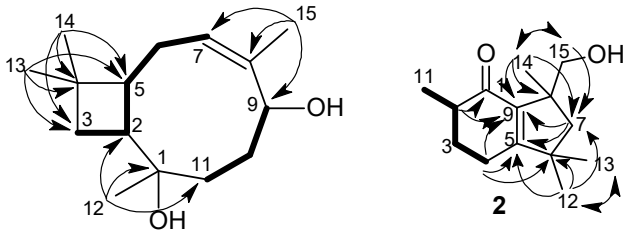

Figure 2: Key COSY (bold lines) and HMBC (arrows) correlations for **1**.

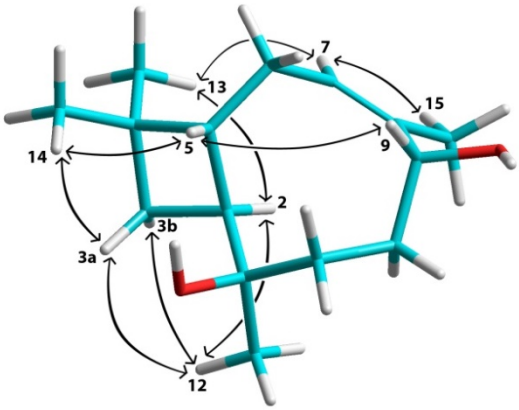

Figure 3: Key ROESY correlations of **1**.

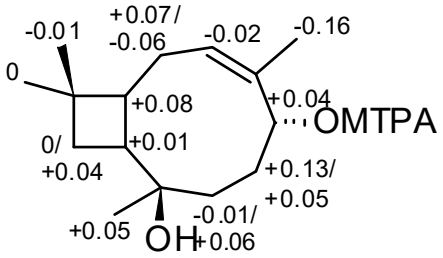

Figure 4: $\Delta\delta^{SR}$ values (in ppm) for each proton indicating the absolute configuration of rickicaryophyllane A (**1**).

aqueous methanol and dichloromethane. Its molecular formula was determined to be $C_{14}H_{22}O_2$ by HRESIMS data, implying four degrees of unsaturation. Proton and ^{1}H,^{1}H HSQC NMR spectra showed the presences of four methyls, four methylenes (one of them oxygenated), and one methine. Carbon and ^{1}H,^{13}C HMBC NMR spectra revealed the further presence of one conjugated ketone, two sp^2, and two sp^3 hybridized quaternary carbons. ^{1}H,^{1}H COSY and TOCSY spectra starting from methyl H_3-11 assembled the spin system H_3-11/H-2/H_2-3/H_2-4 (see Figure 2). HMBC correlations from H-2 to C-1/C-9 and H_2-4 to C-5/C-9 completed the 6-membered ring moiety. HMBC correlations from geminal methyls H_3-12 and H_3-13 to C-5/C-6/C-7, H_2-7 to C-5/C-6/C-8/C-9 respectively, and H_3-14 and H_2-15 to C-7/C-8/C-9 revealed the five membered ring and simultaneously established the planar structure of **2**.

Structurally, compound **2** is an unprecedented 10-nor derivative of the botryane sesquiterpenoid family. Since the absolute configuration of botrydial has been demonstrated on the basis of total synthesis [14], we propose the 2*R*,8*S* configuration for **2**, which is in common with the botryane type sesquiterpenoids.

Metabolite **3** accrued in the course of the isolation of **2** from the dichloromethane crude extract. From its HRESIMS, ^{1}H and ^{13}C NMR data it was recognized as orcacetophenone (**3**), which is known as a natural product from the fungus *Scolecotrichum graminis* [15] and the tree *Syzygium discolor* [16]; furthermore, it was observed as a shunt product in a heterologous expression approach for polyketide genes of *Streptomyces* [17].

The new compounds **1** and **2** were tested for activity against various Gram-positive and Gram-negative bacteria, as well as fungi, in addition to possible cytotoxicity [18], but no activity was observed. In conclusion, the isolation of the new metabolites **1** and **2** raises the number of novel metabolites from *H. rickii*, which belong to six structural backbones including terphenyls as well as botryane, abietane, 14-noreremophilane, silphiperfolane and caryophyllene terpenoids, an uncommonly broad structural variety.

Experimental

General: Optical rotations were determined with a Perkin-Elmer 241 spectrometer and UV spectra with a Shimadzu UV-Vis spectrophotometer UV-2450. NMR spectra were recorded with a Bruker Avance III 700 spectrometer with a 5 mm TCI cryoprobe (^{1}H 700 MHz, ^{13}C 175 MHz) and an Avance III 500 (^{1}H 500 MHz, ^{13}C 125 MHz) spectrometer. HRESIMS were obtained with an Agilent 1200 series HPLC-UV system [column 2.1 x 50mm, 1.7 µm, C_{18} Acquity UPLC BEH (Waters), solvent A: H_2O + 0.1 formic acid; solvent B: ACN + 0.1% formic acid, gradient: 5% B for 0.5 min, increasing to 100% B in 19.5 min, maintaining 100% B for 5 min, flow rate 0.6 mL/min, UV/Vis detection 200-600 nm] combined with an ESI-TOF-MS (MaXis, Bruker) [scan range

100-2500 *m/z*, rate 2 Hz, capillary voltage 4500 V, dry temperature 200°C]. 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 either a VP Nucleodur C18 ec column (250 x 21 mm, 7 µm, Macherey-Nagel) or a VP Nucleodur C18 ec column (125 × 40 mm, 7 µm; Macherey-Nagel) was used. The mobile phase was composed of deionized water (Milli-Q, Millipore, Schwalbach, Germany) with 0.05 % trifluoroacetic acid (solvent A1; Roth) and acetonitrile (ACN) with 0.05 % trifluoroacetic acid (solvent B1). Flow rate was set to 40 or 15 mL/min depending on the stationary phase.

Fungal material: Stromata (fruiting bodies) of *Hypoxylon rickii* MJF10324 were collected in 2010 from the Caribbean island of Martinique by J. Fournier. The strain was designated as the epitype of the species and has been deposited in public culture collections (MUCL 53309, CBS 129345) [4].

Cultivation on 70 L scale and isolation of 1: Large-scale fermentation of *H. rickii* was described previously [4]. The fermentation was aborted after 7 days as sugars (sucrose, fructose) were depleted. After separation from the biomass, the supernatant was incubated with 1 kg adsorbent resin (Amberlite^{TM} XAD 16N), followed by an overnight elution with 5 L MeOH. The eluate was evaporated yielding 40 g of crude extract of which a fraction (10 g) was used to obtain compounds **1** - **3**. The crude extract was dissolved in 1 L of 15% H_2O, 85% MeOH and extracted with 1L heptane. The heptane phase was separated and evaporated to yield 385 mg crude extract, while the polar phase was set to 70% H_2O, 30% MeOH and extracted with 1L dichloromethane (DCM). The DCM phase was separated and evaporated to yield 4.3 g crude extract.

The heptane extract was further separated twice by preparative RP HPLC. For the first separation a flowrate of 40 mL/min was used with the following gradient: linear from 45% to 100% B in 30 min, isocratic at 100% for 5 min. Compound **1** was purified from a fraction [retention time (RT) 11 min] using a linear gradient from 60% - 85% B in 30 min, a linear gradient to 100% in 5 min and isocratic conditions for 5 min whilst applying a flowrate of 15 mL/min. A fraction (1.1 mg) at a RT of 8 min yielded the pure compound.

To isolate compounds **2** and **3**, the DCM extract was separated using a Reveleris® X2 Flash Chromatography System (W. R. Grace & Co) equipped with a Reveleris® Silica 40 g cartridge, DCM as solvent A and acetone as solvent B with a flowrate of 40 mL/min. The following gradient was applied: 0% B for 2 min, linear to 7% B in 10 min, linear to 16% B in 10 min, isocratic for 10 min, linear to 100% B in 1.4 min, isocratic for 14.3 min. A fraction (205 mg) with a RT of 3.7 -6.6 min was further separated by preparative RP-HPLC with a flowrate of 40 mL/min and a linear gradient from 35% - 75% B in 30 min, a linear gradient to 100% in 3 min and isocratic conditions for 10 min. A fraction with a RT of 8 min yielded 1.3 mg of compound **3**. A second fraction (183 mg) from the flash chromatography separation with a RT of 25.7 – 33.0 min was purified twice by preparative RP-HPLC. First a flowrate of 40 mL/min was used with linear gradient from 20% - 80% B in 30 min, linear to 100% B in 5 min and isocratic conditions for 5 min. A fraction (31.5 mg) with a RT of 7- 9 min was further separated with unacidified solvents and the following conditions: Flowrate of 15 mL/min, linear from 60% - 80% B in 35 min, linear to 100% B in 1 min, isocratic for 4 min. A single fraction collected at 12.5 min yielded 1.4 mg of compound **2**.

Rickicaryophyllane [**(1*S*,2*R*,5*R*,6*Z*,9*R*)-2,6,10,10-tetramethylbicyclo[7.2.0]undec-6-ene-2,5-diol**] (**1**)
Colorless oil, 1.0 mg
$[\alpha]_D$: -23 (*c* 0.1, $CHCl_3$).
Rt: 9.7 min (HRESIMS).
^{1}H NMR (700 MHz, CD_3OD): Table 1.
^{13}C NMR (125 MHz, CD_3OD): Table 1.
ESIMS: *m/z* [M+Na^+] 261.20; [M+H^+-H_2O] 221.19; [M+H^+-2H_2O] 203.20.
HRESIMS: *m/z* [M + Na^+] calcd for $C_{15}H_{26}O_2Na$: 261.1825; found: 261.1825; [M+H^+-H_2O] calcd for $C_{15}H_{25}O$: 221.1900; found: 221.1901.

Metabolite 2 [**(3*S*,5*R*)-3-(hydroxymethyl)-1,1,3,5-tetramethyl-1,2,3,5,6,7-hexahydro-4*H*-inden-4-one**]
Colorless oil, 1.4 mg
Rt: 9.1 min (HRESIMS).
UV (ACN/H_2O): 252 nm.
^{1}H NMR (700 MHz, $CDCl_3$): δ_H 3.47 (1H, d, *J* = 11.0 Hz, H_a-15), 3.38 (1H, d, *J* = 11.0 Hz, H_b-15), 2.38 (1H, m, H-2), 2.30 (2H, m, H_2-4), 2.10 (1H, m, H_a-3), 1.71 (1H, m, H_b-3), 1.62 (1H, m, H_a-7), 1.55 (1H, m, H_b-7), 1.23 (3H, s, H_3-14), 1.16 (3H, s, H_3-12), 1.13 (3H, d, *J* = 6.8 Hz, H_3-11), 1.11 (3H, s, H_3-13).
^{13}C NMR (175 MHz, CD_3OD $CDCl_3$): δ_c 203.7 (C, C-1), 172.8 (C, C-5), 140.1 (C, C-9), 71.7 (CH_2, C-15), 51.0 (CH_2, C-7), 49.1 (C, C-8), 45.3 (C, C-6), 42.0 (CH, C-2), 31.4 (CH_2, C-3), 28.4 (CH_3, C-12), 28.3 (CH_3, C-13), 23.4 (CH_3, C-14), 22.4 (CH_2, C-4), 15.0 (CH_3, C-11).
ESIMS: *m/z* [M+Na^+] 245.19; [M+H^+] 223.20; [M+H^+-H_2O] 205.18.
HRESIMS: *m/z* [M + H^+] calcd for $C_{14}H_{23}O_2$: 223.1693; found: 223.1688.

Orcacetophenone [**1-(2,4-dihydroxy-6-methylphenyl)ethanone**] (**3**)
Colorless oil, 1.3 mg
Rt: 5.4 min (HRESIMS).
^{1}H NMR (500 MHz, $CDCl_3$): δ_H 13.41 (1H, br s, O*H*), 6.24 (1H, d, *J* = 2.5 Hz, H-5), 6.22 (1H, d, *J* = 2.5 Hz, H-7), 2.54 (3H, s, H_3-9).
^{13}C NMR (175 MHz, $CDCl_3$): δ_c 204.1 (C, C-2), 166.9 (C, C-4), 160.8 (C, C-6), 142.8 (C, C-8), 115.7 (C, C-3), 111.8 (CH, C-7), 101.9 (CH, C-5), 33.2 (CH_3, C-1), 25.2 (CH_3, C-9.
ESIMS: *m/z* [M+H^+] 167.11; [M+H^+-H_2O] 149.09, [M-H^-] 164.99.
HRESIMS: *m/z* [M + H^+] calcd for $C_9H_{11}O_3$: 167.0703; found: 167.0702; [M+H^+-H_2O] calcd for $C_9H_9O_2$: 149.0597; found: 149.0593.

Synthesis of (R)- and (S)-MTPA esters of 1: For the preparation of the (*R*)-MTPA ester, 0.2 mg of **1** was dissolved in 600 µL of pyridine-d_5, and 10 µL of (*S*)-MTPA chloride was added. The mixture was kept at 25°C for 30 min before ^{1}H, COSY and TOCSY NMR spectra were measured.
^{1}H NMR (pyridine-d_5, 700 MHz): δ 6.23 (dd, *J* = 9.7, 3.9 Hz, H-9), 5.63 (dd, *J* = 9.0, 8.0 Hz, H-7), 2.69 (m, H-5), 2.49 (m, H_β-6), 2.22 (m, H_β-10), 2.15 (ddd, *J* = 14.5, 8.0, 7.0 Hz, H_α-6), 2.05 (t, *J* = 9.9 Hz, H_α-3), 1.96 (m, H-2), 1.92 (ddd, *J* = 14.5, 6.6, 3.0, H_α-11), 1.83 (s, H_3-15), 1.79 (m, H_α-10), 1.75 (m, H_β-11), 1.52 (dd, *J* = 9.9, 9.0, H_β-3), 1.15 (s, H_3-12), 1.03 (s, H_3-13), 1.01 (s, H_3-14).

The (*S*)-MTPA ester was prepared in the same manner by the addition of 10 µL of (*R*)-MTPA chloride:
^{1}H NMR (pyridine-d_5, 700 MHz): δ 6.27 (dd, *J* = 9.7, 3.9 Hz, H-9), 5.61 (t, *J* = 7.1 Hz, H-7), 2.77 (m, H-5), 2.56 (m, H_β-6), 2.35 (m, H_β-10), 2.09 (m, H_α-6), 2.05 (m, H_α-3), 1.97 (m, H-2), 1.91 (m,

H_α-11), 1.84 (m, H_α-10), 1.81 (m, H_β-11), 1.67 (s, H_3-15), 1.56 (t, *J* = 8.1 Hz, H_β-3), 1.20 (s, H_3-12), 1.03 (s, H_3-13), 1.00 (s, H_3-14).

Supplementary data: ESIMS and HRESIMS, ^1H, ^{13}C, COSY, HSQC, HMBC, ROESY NMR spectra of **1 - 3** can be found as Supplementary data.

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