Ascomycones A−C, Heptaketide Metabolites from an Unidentified Ascomycete

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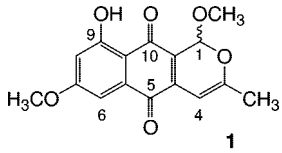
Received September 11, 2008

The ascomycones A−C (**1**−**3**), three novel heptaketide-derived secondary metabolites, have been isolated from cultures of an unknown ascomycete. While **1** and **2** are closely related pyranonaphthoquinones, **3** has a bicyclic dihydronaphthoquinone core. Compounds **2** and **3** exhibited significant activity against several phytopathogenic fungi.

The intensely red colored culture filtrates of a hitherto unidentified ascomycete exhibited activity against the notorious phytopathogens *Magnaporthe grisea* (rice blast fungus) and *Fusarium graminearum* (wheat head blight fungus). Here, we report the isolation and characterization of three novel heptaketidic metabolites (**1**−**3**), which we named ascomycones A−C, that are responsible for this biological activity.

The ascomycete was collected in French Guiana, where it was found on the bark of a dead twig. It was grown on HMG medium at ambient temperature until the glucose was consumed (14 days). The mycelia were removed by filtration, and the culture filtrate (15 L) was extracted with ethyl acetate. Removal of the solvent *in vacuo*, fractionation, and purification by silica gel column chromatography (CC) furnished three fractions, two of which were further purified by preparative HPLC, while the third, insoluble fraction was purified by several washing steps. Compounds **1** (6.8 mg), **2** (29.5 mg), and **3** (117.8 mg) were thus obtained in pure form.



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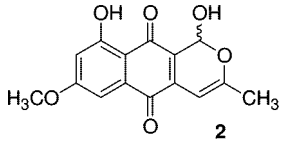

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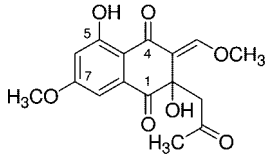

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The structure of **1** (dark red crystals, mp 81−82 °C), the least polar of the three compounds, was elucidated by a combination of spectroscopic techniques. APCI-MS and ESI-HRMS indicated a composition of $C_{16}H_{14}O_6$, requiring 10 degrees of unsaturation. NMR spectroscopy (^{1}H, ^{13}C, COSY, HSQC, HMBC) (Table 1) indicated the structure to be that of a 5,7-disubstituted naphthoquinone annellated to a 6-methyl-substituted 1-methoxy-1*H*-pyran. The gs-HMBC spectrum recorded under standard conditions (refocusing delay set to 62.5 ms, corresponding to $^nJ_{C,H} = 8$ Hz) showed unusually long reaching correlations over up to six bonds. The tricyclic core was determined to be that of a 1,3,7,9-tetrasubstituted 1*H*-benzo[*g*]isochromene-5,10-dione. This extended chromophore is responsible for the intense absorption of compound **1** in the UV and visible range. The UV/vis spectrum of a methanolic solution showed five absorption maxima above 200 nm with the one at 482 nm (log $\varepsilon = 3.66$) being responsible for the red color. The structural assignment based on spectroscopic data was confirmed by X-ray crystallography (Figure 1). Compound **1** was named ascomycone A.

Compounds **1** and **2** had highly similar UV spectra. Compound **2** (ascomycone B, dark red amorphous solid) had an elemental composition of $C_{15}H_{12}O_6$, and its ^{1}H and ^{13}C chemical shifts differed only slightly from those of **1**, with the exception of the 1-OCH_3 resonance, which was absent in compound **2**. Instead, an OH resonance with heterocorrelations to C-1 and C-10a was observed at δ 4.88 (Table 1), indicating that **2** was a hemiacetal with a free 1-OH group.

Compound **3** (ascomycone C, $C_{16}H_{16}O_7$, slightly pinkish crystals, mp 144−145 °C dec) was the most polar of the three compounds. Although the UV/vis spectrum was quite different from those of compounds **1** and **2**, one- and two-dimensional NMR experiments revealed the western half of the molecule to be identical to that of the other ascomycones. In contrast, **3** did not contain a pyran ring. Carbon atoms 3 and 4 of compound **3** were part of a 2-propanone chain, while C-1 and the 1-methoxy group of **1** were contained in a methyl enol ether moiety. The absence of a double bond connecting the two central carbonyl groups in **3** is responsible for the lack of absorption in the visible range. In addition, the HMBC correlations measured under the same conditions as for the other ascomycones were much less far-reaching, with four bonds being the maximum distance. While **1** and **2** did not show detectable optical rotation, **3** was dextrorotatory ($[\alpha]_D^{25}$ +25.5, *c* 0.67, CH_3CN). The configuration of the exocyclic double bond could not be determined by NOE measurements, as the saturation of neither the methoxy resonance nor the olefinic signal enhanced the intensity of the signals of the propanone residue. Finally, X-ray crystallographic analysis revealed the *E*-configuration of the enol ether; however, the acquired data did not allow determination of the absolute configuration of the stereogenic center at C-2 (Figure 2).

To convert **3** to its tricyclic congener **1**, closure of the pyran ring and a dehydration step would be required. The reverse process could involve opening of the pyran ring under tautomerization to the ketone and hydration of the C2−C3 bond. Although neither the biogenetic relationship of **1** and **3** nor the exact biogenetic

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10.1021/np800570w CCC: $40.75
Published on Web 11/07/2008

Table 1. ^{13}C and ^{1}H Chemical Shifts (ppm, $CDCl_3$) of Ascomycones A and B (**1** and **2**)

	ascomycone A (**1**)			ascomycone B (**2**)[a]	
position	δ_C, mult.	δ_H (*J* in Hz)	HMBC	δ_C, mult.	δ_H (*J* in Hz)
1	95.1, CH	6.25, s	1-OCH_3 (**1**), 3, 4 (**1**), 4a, 10, 10a	87.4, CH	6.48, d (7.0)
3	162.3, qC			162.1, qC	
4	94.6, CH	6.14, q (0.8)	3, 3-CH_3, 5, 10, 10a	93.5, CH	6.00, q (0.9)
4a	137.1, qC			136.4, qC	
5	182.4, qC			182.1, qC	
5a	133.1, qC			132.8, qC	
6	107.9, CH	7.19, d (2.5)	5, 5a, 7, 8, 9a	107.3, CH	7.06, d (2.5)
7	165.4, qC			165.0, qC	
8	106.7, CH	6.64, d (2.5)	6, 7; 9, 9a	106.2, CH	6.55, d (2.5)
9	163.9, qC			163.5, qC	
9a	109.8, qC			109.3, qC	
10	186.1, qC			185.8, qC	
10a	120.8, qC			121.1, qC	
1-OCH_3	55.8	3.61, s	1		
1-OH			1, 10a		4.88, mc
3-CH_3	20.8	2.29, d (0.8)	3, 4, 4a, 10, 10a	20.7	2.06, d (0.9)
7-OCH_3	55.9	3.89, s	7	55.6	3.80, s
9-OH		12.45, s	not determined		12.41, s

[a] Solvent $CDCl_3$ + 30% CD_3CN.

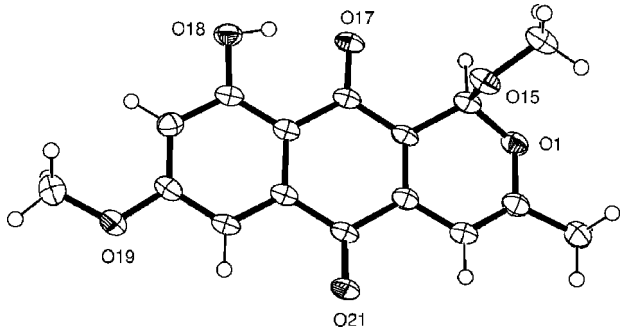

Figure 1. Crystal structure of **1** at 193 K (OTREP plot). Thermal ellipsoids are drawn at the 50% probability level.

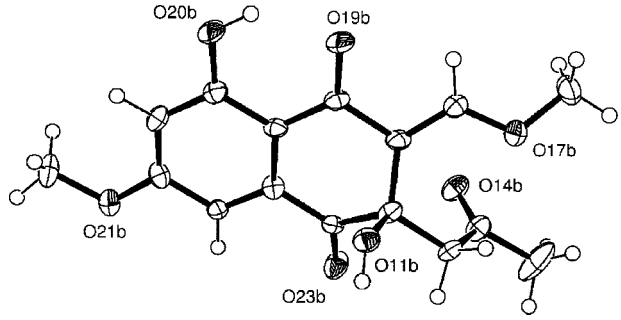

Figure 2. Crystal structure of **3** at 193 K (OTREP plot). Thermal ellipsoids are drawn at the 50% probability level.

pathway of the ascomycones is known, their structural similarity to 6-*O*-demethyl-5-deoxyanhydrofusarubin,[1] dehydroherbarin,[2] 1-hydroxydehydroherbarin,[3] thysanone,[4] and other pyranonaphthoquinones[5] suggests a heptaketide origin according to the folding mode F[6], which would require the oxidation of C-5 (or C-1 in **3**) at a later stage.

All three compounds exhibited pronounced biological activities. Their antimicrobial and cytotoxic activities are summarized in Table 2. As can be seen, ascomycone B (**2**) was the most active antifungal compound followed by ascomycone C (**3**). The latter was the least cytotoxic. Gram-positive bacteria were more sensitive toward ascomycone A (**1**) as compared to the other two compounds. The Gram-negative bacteria *E. coli, Enterobacter dissolvens*, and *Proteus vulgaris* were not senstive up to 50 μg/mL. None of the compounds exhibited phytotoxic activity in a seed germination and growth test with *Lepidium sativum* and *Setaria italica* up to 300 μg/mL, and the nematodes *Caenorhabditis elegans* and *Meloidogyne incognita* were not affected by 100 μg/mL of the compounds.

Experimental Section

General Experimental Procedures. Melting points were measured on a Dr. Tottoli apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer 241 polarimeter at 25 °C. IR and UV spectra were measured with a Bruker IFS48 FTIR spectrometer and a Perkin-Elmer Lambda-16 spectrophotometer, respectively. ^{1}H NMR (400 MHz) and ^{13}C NMR (100.6 MHz) were recorded at 25 °C with a Bruker Avance-II spectrometer equipped with an inverse multinuclear 5 mm probehead and a z-gradient coil. The spectra were measured in $CDCl_3$, and the chemical shifts were referenced to the residual solvent signal ($CDCl_3$: δ_H = 7.26 ppm, δ_C = 77.0 ppm, CD_3CN: δ_H = 1.94 ppm, δ_C = 1.32 ppm). Standard pulse sequences for gs-COSY, gs-HSQC, and gs-HMBC, and gs-NOESY experiments were used. The refocusing delays for the inverse heterocorrelations were set to 3.45 and 62.5 ms, corresponding to $^{1}J_{C,H}$ = 145 Hz and $^{n}J_{C,H}$ = 8 Hz, respectively. Processing of the data was performed with the Mestre-C Software (Mestrelab Research). APCI-MS spectra were measured from a solution of the analyte in acetonitrile/water with a Hewlett-Packard MSD 1100 using an evaporator temperature of 400 °C and a drying gas temperature of 350 °C at a flow of 6 L/h (N_2). In positive ionization mode, the capillary voltage was 3.5 kV, and the corona discharge current was 4 μA. In negative ionization mode, the capillary voltage amounted to 2.2 kV, and the corona discharge current was 6 μA. ESI-HRMS data were measured from a solution of the analyte in acetonitrile with a Waters Q-TOF-Ultima 3 equipped with a LockSpray interface (tri-*n*-heptylamine as external reference).

Organism. The millimeter-sized, orange-colored, disk-shaped fruiting bodies of the producing organism were growing on the bark of a dead twig collected in French Guiana. The dried fruiting bodies in the herbarium material that is kept at the IBWF unfortunately contained no ripe asci with mature ascospores. Mycelial cultures were obtained from ascospores of the fresh material. The whitish mycelia became dark gray in older cultures, and the surrounding agar turned purple red from the pigments produced. Since the formation of conidia was not observed, the identification of the strain by molecular taxonomy was performed. Nuclear DNA was extracted from living mycelium, and the intertranscribed spacers 1 and 2 as well as the 5.8S region of the rDNA were PCR-amplified, purified, and sequenced. Similarity searches in nonredundant GenBank and EMBL indicated similarity to ascomycetes (highest scores ascomycete sp. VegaE4-32, accession number EU002909 and fungal sp. EXPO528F, accession number DQ914703). The DNA sequences of the producing strain are deposited in GenBank, accession number FJ375933. Mycelial cultures are deposited as IBWF 77-89A at the IBWF in Kaiserslautern, Germany. The cultures are maintained on HMG agar and are stored at 4 °C.

Fermentation and Isolation. The producing organism was cultivated in HMG medium (15 L) containing per liter of H_2O: glucose 10 g, malt extract 10 g (Dr. Fränkle and Eck, Fellbach), yeast extract 4 g

Table 2. Biological Activities of Ascomycones A−C (**1**−**3**)[a]

biological activity	ascomycone A (**1**)	ascomycone B (**2**)	ascomycone C (**3**)
Conidia germination	IC_{100} (μg/mL)	IC_{100} (μg/mL)	IC_{100} (μg/mL)
Magnaporthe grisea	50	1.0	2.5
Fusarium graminearum	50	0.5	2.5
Botrytis cinerea	>50	5.0	50
Phytophthora infestans	>50	5.0	5.0
Penicillium notatum	>50	10	15
Paecilomyces variotii	>50	>50	>50
Mucor miehei	50	10	>50
Agar diffusion assay	inhibition zone (mm)	inhibition zone (mm)	inhibition zone (mm)
Bacillus brevis	14	11	7
Bacillus subtilis	11	10	0
Nematospora coryli (yeast)	0	13	11
Serial dilution test	MIC (μg/mL)	MIC (μg/mL)	MIC (μg/mL)
E. coli K12	>50	>50	>50
Enterobacter dissolvens	>50	>50	>50
Proteus vulgaris	>50	>50	>50
Cytotoxic activity	IC_{50} (μg/mL)	IC_{50} (μg/mL)	IC_{50} (μg/mL)
HeLa S3 cells	5.0−10.0	5.0	50

[a] Antimicrobial activities measured in a germination assay using fungal conidia, in the serial dilution test or in the disk diffusion assay with 50 μg per filter disk (6 mm diameter). Cytotoxic activity was determined in a cell proliferation assay.

(Hartge, Hamburg). Fermentation was carried out in a 20 L fermenter (Biostat A-20, Braun, Melsungen) at 22−24 °C; 200 mL of a well-grown culture in the same medium was used as inoculum. After 14 days when the glucose was used up, the culture fluid was separated from the mycelia by filtration. The culture fluid was extracted with ethyl acetate (10 L) and the organic phase concentrated *in vacuo*. The crude extract (3.4 g) was applied onto a silica gel column (Merck 60, 0.063−0.2 mm). Elution with cyclohexane/ethyl acetate (4:1) resulted in an intermediate product (68.8 mg), which was further purified by preparative HPLC (Merck Lichrospher RP18, 5 μm, column 25 × 250 mm, flow 20 mL/minute; isocratic MeCN/H_2O, 60:40), yielding **1** (6.8 mg). Elution of the silica column with cyclohexane/ethyl acetate (7:3) yielded a second intermediate product (197.6 mg), which was purified by washing with ice cold methanol (4 × 3 mL) and ice cold ethyl acetate (3 mL) to give **2** (29.5 mg). Elution with cyclohexane/ethyl acetate (3:7) furnished a third intermediate product (686.2 mg), which was further purified by preparative HPLC (Machery & Nagel Polygoprep 100-20 C18 15−25 μm, 15 g in a column with 20 mm diameter, stepwise elution with H_2O/MeCN, 90:10, 70:30, and 50:50) to give an enriched material (285.0 mg). Washing with ice cold methanol (3 × 3 mL) and ice cold ethyl acetate (3 mL) yielded pure **3** (117.8 mg).

Biological Assays. Inhibition of germination of fungal conidia was tested as described previously.[7] Antimicrobial activity was determined using serial dilution and agar diffusion assays. Nematicidal activity and inhibition of germination and growth of seeds of *Setaria italica* and *Lepidium sativum* were tested as described.[8] Bacteria were tested in nutrient broth (Difco); yeasts and fungi, in HMG medium or agar medium respectively.

Cytotoxic activity was assayed as described previously[9] with slight modifications. HeLa S3 (ATCC CCL 2.2) cells were grown in D-MEM (GIBCO, BRL), supplemented with 10% fetal calf serum (GIBCO, BRL), 65 μg/mL of penicillin G, and 100 μg/mL of streptomycin sulfate. The assays contained 1×10^5 cells/mL medium. The concentration at which cell proliferation was reduced by 50% is given as the IC_{50} value.

Ascomycone A, 9-hydroxy-1,7-dimethoxy-3-methyl-1*H*-benzo[*g*]isochromene-5,10-dione (1): red crystalline solid, mp 81−82 °C; UV (MeOH) λ_{max} (log ε) 216 (4.35), 256 (4.06), 276 (4.05), 349 (3.54), 482 (3.66); IR (KBr) ν_{max} 3437 (broad), 2937, 2840, 1648, 1612, 1595, 1566, 1443, 1389, 1352, 1303, 1288, 1253, 1211, 1096, 1084, 944, 809 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HMBC contacts (in order of decreasing cross-peak intensity) 6-H with C-9a, C-5, C-8, C-7, C-5a; 8-H with C-6, C-9a, C-9, C-7; 1-H with 1-OCH_3, C-3, C-4a, C-10a, C-10, C-4; 4-H with C-10a, C-3, C-5, 3-CH_3, C-10; 7-OCH_3 with C-7; 1-OCH_3 with C1, 3-CH_3 with C-3, C-4, C-10a, C-4a, C-10; APCI-MS (negative) *m/z* 302.1 $[M]^-$ (100), 287.0 (25), 270.0 (33), 269.0 (31), 260.0 (34); APCI-MS (positive) *m/z* 270.9 $[M - OCH_3]^+$ (100); ESI-HRMS *m/z* 325.0689 (calcd for $C_{16}H_{14}O_6Na$, 325.0688).

Ascomycone B, 1,9-dihydroxy-7-methoxy-3-methyl-1*H*-benzo[*g*]isochromene-5,10-dione (2): amorphous red solid, decomposition >175 °C; UV (MeOH) λ_{max} (log ε) 216 nm (4.40), 259 (4.11), 277 (4.14), 349 (3.67), 483 (3.73); IR (KBr) ν_{max} 3352 (br), 2948, 2836, 1676, 1648, 1617, 1561, 1447, 1391, 1305, 1089, 1030, 938, 843, 771 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HMBC contacts (in order of decreasing cross-peak intensity) 6-H with C-9a, C-5, C-8, C-7, C-5a; 8-H with C-9a, C-6, C-9, C-7; 1-H with C-3, C-4a, C-10a, C-10; 4-H with C-3, C-10a, C-5, 3-CH_3, C-10; OH with C-10a, C-1; OCH_3 with C-7; 3-CH_3 with C-3, C-4, C-10a, C-4a, C-10; APCI-MS (negative) *m/z* 288.0 $[M]^-$ (64), 287.0 $[M - H]^-$ (100), 269.0 (61); APCI-MS (positive) *m/z* 271.0 $[M - OH]^+$ (100); ESI-HRMS *m/z* 311.0542 (calcd. For $C_{15}H_{12}O_6Na$, 311.0532).

Ascomycone C, (*E*)-2,5-dihydroxy-7-methoxy-3-methoxymethylene-2-(2-oxopropyl)-2,3-dihydro[1,4]naphthoquinone (3): slightly pink crystals, mp 144−145 °C (dec); $[\alpha]^{25}_D$ +25.5 (*c* 0.67, CH_3CN); UV (MeOH) λ_{max} (log ε) 250 (3.72), 355 (3.41); IR (KBr) ν_{max} 3432 (br), 3367 (br), 3087, 2948, 2853, 1705, 1644 (sh), 1580, 1444 (sh), 1389 (sh), 1254, 1204, 1160, 1138, 986, 866 (sh), 606 cm^{-1}; ^1H NMR (400 MHz, CD_3CN) δ 13.49 (s, 1H, 5-OH), 7.76 (s, 1H, =C*H*−OCH_3), 7.07 (d, ^4J = 2.7 Hz, 1H, 8-H), 6.73 (d, ^4J = 2.7 Hz, 1H, 6-H), 4.11 (s, 1H, 2-OH), 4.01 (s, 3H, β-OCH_3), 3.89 (s, 3H, 7-OCH_3), 3.71 (d, ^2J = 17.5 Hz, CH_2-H_a), 3.44 (d, ^2J = 17.5 Hz, CH_2-H_b), 2.05 (s, 3H, $COCH_3$); ^{13}C NMR, HSQC, HMBC (100.6 MHz, CD_3CN) δ 208.4 ($COCH_3$), 195.1 (C-1), 190.2 (C-4), 166.8 (C-7), 165.9 (C-5), 165.6 (=*C*H−OCH_3), 136.0 (C-8a), 116.4 (C-3), 112.8 (C-4a), 107.4 (C-6), 106.3 (C-8), 73.6 (C-2), 64.5 (β-OCH_3), 56.9 (7-OCH_3), 52.1 (CH_2), 30.2 ($COCH_3$); HMBC contacts 5-OH with C-4a, C-5, C-6; =C*H*−OCH_3 with β-O*C*H_3, C-3, C-2, C-4; 8-H with C-4a, C-1, C-6; 6-H with C-5/C-7, C-4a, C-8; 2-OH with C-3, C-2, CH_2, C-1; β-O*C*H_3 with =*C*H−OCH_3; 7-OCH_3 with C-7; $CH_2-H_{a/b}$ with C-2, *C*OCH_3, C-1, C-3; CO*C*H_3 with *C*OCH_3, CH_2, C-2; APCI-MS (negative) *m/z* 319.1 $[M - H]^-$ (100), 275.0 (23), 247.0 (37), 219.0 (37), 269.0 (31), 260.0 (34); APCI-MS (positive) *m/z* 289.0 $[M - OCH_3]^+$ (41), 271.0 $[M - OCH_3 - H_2O]^+$ (100), 261.1 (39), 247.0 (68); ESI-HRMS *m/z* 343.0799 (calcd for $C_{16}H_{16}O_7Na$, 343.0794).

Crystal data for **1**: formula $C_{16}H_{14}O_6$, triclinic, space group *P*1, *a* = 7.993(1) Å, α = 86.32(3)°, *b* = 8.766(2) Å, β = 80.07(2)°, *c* = 10.816(4) Å, γ = 63.37(1)°, *V* = 667.3(3) $Å^3$, *z* = 2, *D* = 1.504 g cm^{-3}. *T* = 193 K, *R* = 0.0764, *Rw* = 0.2314. Crystal data for **3**: formula $C_{16}H_{16}O_7$, monoclinic, space group $P2_1$, *a* = 8.0031(4) Å, *b* = 17.5926(6) Å, β = 107.005(5)°, *c* = 10.8678(6) Å, *V* = 1463.2(1) $Å^3$, *z* = 4, *D* = 1.454 g cm^{-3}. *T* = 193 K, *R* = 0.0814, *Rw* = 0.2763. CCDC-688240 and CCDC-688239 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge at www.ccdc.cam.ac.uk/conts/retrieving.html [or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: (internat.) +44-1223/336-033; E-mail: deposit@ccdc.cam.ac.uk].

Acknowledgment. We thank Dr. D. Schollmeyer (University of Mainz) for the X-ray crystallographic analysis of ascomycones A and C (**1** and **3**) as well as the Kompetenzzentrum der Integrierten

Naturstoff-Forschung. The expert technical assistance of A. Meffert, N. Heil, and S. Pauls is gratefully acknowledged.

Supporting Information Available: ^{1}H, ^{13}C, and 2D NMR spectra of all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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NP800570W