(Iso)-Quinoline Alkaloids from Fungal Fruiting Bodies of *Cortinarius subtortus*

Axel Teichert, Jürgen Schmidt, Andrea Porzel, Norbert Arnold,* and Ludger Wessjohann

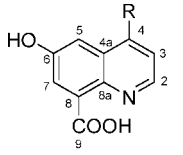
Department of Bioorganic Chemistry, Leibniz Institute of Plant Biochemistry, Weinberg 3, D-06120 Halle/Saale, Germany

Received February 6, 2008

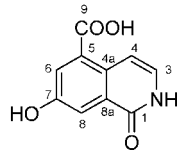
Chemical analysis of the fruiting bodies of the agaricoid fungus *Cortinarius subtortus* yielded three new natural products, two quinoline and one isocarbostyryl alkaloid. The structures of compounds **1**−**3** were determined by analysis of NMR and MS data. Compound **1** exhibited inhibitory effects against the phytopathogenic fungus *Colletotrichum coccodes*. All three compounds displayed moderate antioxidant activity in a DPPH free radical scavenging bioassay.

Cortinarius subtortus (Pers.) Fr. belongs to the subgenus *Phlegmacium* in *Cortinarius.* According to *Index fungorum* this genus comprises the most epithets in Agaricales.[1] The subgenus *Phlegmacium* is characterized by a more or less viscid cap compared with a dry stalk. *C. subtortus* is found fruiting in groups or clusters on the ground among mosses in bogs and conifers, but rarely in deciduous forests. The ochraceous cap with olivaceous tint is 1.5−6 cm wide, bell-shaped when young, becoming broadly convex in age. The pale ochraceous stalk is 4−9 cm long and 0.4−1.5 cm wide, becoming enlarged downward. With its olivaceous colors appearing especially at the gills when young, its bitter taste, and characteristic smell of cedar wood, this fungus is unmistakeable.[2] Molecular analysis based on the ITS and 5.8S regions of nuclear rDNA sequences leads to the incorporation of *C. subtortus* together with *C. infractus* in the section Infracti.[3] This is in opposition to the classification based on microscopic characters, which incorporates *C. subtortus* in the section Subtorti.[4] *C. infractus* produces the indole alkaloids infractine, 6-hydroxyinfractin, and infractopicrin responsible for the bitter taste of the toadstool and the blue-greenish fluorescence of its MeOH extract under UV_{366nm}.[5] In a very recently reported study neither β-carboline-1-propionic acid nor infractopicrine was found in the crude EtOH extract of *C. subtortus* by comparing LC-MS runs with those of the crude EtOH extract of *C. infractus*, leading to the conclusion to classify these species into different sections.[6]

In continuation of our investigations on the secondary metabolites of fruiting bodies of *Cortinarius* spp. we concentrated on the constituents of *C. subtortus* being responsible for the strong blue-greenish fluorescence of the MeOH extract, which resulted in the isolation and structural elucidation of the new natural products 6-hydroxyquinoline-8-carboxylic acid (**1**), 4-amino-6-hydroxyquinoline-8-carboxylic acid (**2**), and 7-hydroxy-1-oxo-1,2-dihydroisoquinoline-5-carboxylic acid (**3**). Previously, compound **1** was mentioned as a synthetic starting material, without any reported spectroscopic data.[7,8] Compounds **2** and **3** are responsible for the blue-greenish fluorescence of the MeOH extract of *C. subtortus* under UV_{366nm}.



1 R = H
2 R = NH_2



3

The frozen fungal fruiting bodies were successively extracted with MeOH. The crude extract was chromatographed consecutively on Diaion HP 20, silica gel RP-2, and Sephadex LH-20 guided by fluorescence (λ_{exc} = 366 nm). The final purification was achieved by preparative RP-18 HPLC.

Compound **1** forms yellow needles and exhibits an orange fluorescence under UV light (λ_{exc} = 366 nm). The molecular formula $C_{10}H_7NO_3$ was deduced from the positive ion ESI-FTICR-MS (*m/z* 212.0319, $[M + Na]^+$). LC-ESI-MS/MS analysis of the $[M + H]^+$ ion (*m/z* 190) showed fragments at *m/z* 172 ($[M + H - H_2O]^+$), *m/z* 144 ($[M + H - H_2O - CO]^+$), and *m/z* 116 ($[M + H - H_2O - 2CO]^+$). The ^{1}H NMR spectrum offered two separate spin systems, where the signals at δ ^{1}H 7.71 (dd, H-3), 8.54 (dd, H-4), and 8.87 (dd, H-2) indicated a 3-fold-substituted aromatic ring. The second spin system consisted of two aromatic *meta* protons, H-5 (d, δ ^{1}H 8.13, *J* 2.8 Hz) and H-7 (d, 7.54, 2.8). ^{13}C NMR data suggested a quinoline derivative substituted with a carboxy and a hydroxy group. Summarizing the data, the structure of **1** was assigned as 6-hydroxyquinoline-8-carboxylic acid. Compound **1** was previously reported in a synthesis of antiallergic 8-(1*H*-tetrazol-5-ylcarbamoyl)quinoline compounds.[7,8]

Compound **2** was isolated as a white, amorphous powder and exhibited a blue-greenish fluorescence under UV light (λ_{exc} = 366 nm). The positive ion ESI-FTICR-MS afforded the molecular formula $C_{10}H_8N_2O_3$ (*m/z* 227.0425, $[M + Na]^+$). Positive ion LC-ESI-MS/MS analysis of the $[M + H]^+$ ion (*m/z* 205) showed fragments at *m/z* 187 ($[M + H - H_2O]^+$), *m/z* 159 ($[M + H - H_2O - CO]^+$), *m/z* 131 ($[M + H - H_2O - 2CO]^+$), and *m/z* 132 ($[M + H - H_2O - CO - HCN]^+$). The ^{1}H NMR spectrum of compound **2** in DMSO-d_6 differed from **1** only in the replacement of an aromatic proton signal with a broad singlet at δ 8.00 that integrated for two protons, which indicated the presence of an amino function. The positions of the OH and carboxy groups on the quinoline system were assigned by HMBC key correlations between H-7 and C-9 and the high-field shift of C-5 (δ 107.9) caused by the electron-releasing OH group in *ortho* position. NOEs from the amino group to H-3 and H-5 were evident in a 2D NMR ROESY experiment. Thus, the structure of **2** was assigned as 4-amino-6-hydroxyquinoline-8-carboxylic acid, the 4-amino derivative of **1**.

Compound **3** was isolated as a light brownish, amorphous residue that also exhibited a strong greenish fluorescence under UV light. The molecular formula $C_{10}H_7NO_4$ was confirmed by negative ion ESI-FTICR-MS (*m/z* 204.0299, $[M - H]^-$). Interpretation of the 1D and 2D NMR data revealed that it was a trisubstituted isoquinoline derivative. The ^{1}H NMR spectrum indicated five proton signals for two different spin coupling systems. The isoquinoline scaffold is characterized by two *meta*-coupled aromatic proton signals at δ 7.75 (d, *J* 3.0 Hz, H-6) and 7.38 (d, 3.0, H-8) and a three-spin system consisting of two aromatic protons at δ 7.85 (dd, H-3) and 5.85 (dd, H-4), and a signal at δ 14.88 (br d) that was assigned to the lactam NH. On the basis of HMBC correlations and the ^{13}C chemical shifts of C-6 (δ 123.6) and C-8 (δ 111.4), the substituents of **3** were assigned as a carboxy group at C-5, a

* Corresponding author, Tel: +49-345-5582-1310. Fax: +49-345-5582-1319. E-mail: Norbert.Arnold@ipb-halle.de.

10.1021/np8000859 CCC: $40.75
Published on Web 05/01/2008

hydroxy group at C-7, and a carbonyl function at C-1, being part of an amide group. These assignments were supported by a loss of 45 amu in the negative ion LC-ESI-MS/MS as a fragment at *m/z* 159, suggesting the loss of formamide ($[M - H - H_2NCHO]^-$). Thus, compound **3** was characterized as 7-hydroxy-1-oxo-1,2-dihydroisoquinoline-5-carboxylic acid.

The isolated metabolites are closely related to a number of biologically active simple quinoline and isoquinoline carboxylic acid derivatives reported previously from plants, bacteria, and animals.[9] Among them zeanic acid from corn steep liquor was reported as a plant growth regulator, and β-acid has been isolated from rice-bran, showing growth promotive effects.[10,11] Kynurenic acid shows neuroactivity, and xanthurenic acid (4,8-dihydroxyquinaldic acid) is known to be excreted by pyridoxine-deficient animals after tryptophan injection. These compounds play a role as targets for drug discovery and development.[12]

6-Hydroxykynurenic acid was previously isolated from tobacco leaves and *Thapsia villosa*.[13,14] *Ephedra* spp. were shown to contain several quinoline carboxylic acid derivatives including 4-hydroxy-6-methoxyquinoline-2-carboxylic acid from *E. pachyclada* and transtorine from *E. transitoria*, which possess antibacterial activity, and ephadralone from *E. alata*.[15–17] Another derivative, 3-hydroxyquinoline-2-carboxylic acid, is described from *Streptomyces griseoflavus*.[18] Quinolobactin (8-hydroxy-4-methoxyquinaldic acid) was detected in *Pseudomonas fluorescens* as a new siderophore.[19] In Macromycetes, the antimalarial compound 4-hydroxymethylquinoline from the wood-rotting fungi *Trametes versicolor* and *Pycnoporus sanguineus* was reported as the first detection of an unoxidized quinoline nucleus in fungi.[20,21] 4-Aminoquinoline has NMDA receptor antagonistic properties, while other derivatives show potent antimalarial activities.[22,23] Carbostyryl-derived natural products are rarely reported in the literature. Recently 4-carbomethoxy-6-hydroxy-2-quinolone from the aleurone layer of anthocyanin-pigmented rice was described to possess moderate antioxidant activity.[24]

The isolated compounds **1**−**3** exhibited strong fluorescence under UV light (λ_{exc}= 373 nm) when dissolved in MeOH (**1**: $\lambda_{max\ em}$ = 454 nm; 581 nm; **2**: $\lambda_{max\ em}$ = 458 nm; **3**: $\lambda_{max\ em}$ = 449 nm).

Compounds **2** and **3** are responsible for the strong blue-greenish fluorescence of the MeOH extract of *Cortinarius subtortus* fruiting bodies. Since the blue-greenish fluorescent compounds differ from those of *C. infractus*, they might be used as a chemotaxonomical marker for the discrimination of these species.

Compounds **1**−**3** were examined in a mycelium growth inhibition assay using the phytopathogenic fungus *Colletotrichum coccodes* (Wallr.) S. Hughes as test organism, being responsible for the black dot disease of potato, which has become an economically important problem in recent years.[25] Compound **1** demonstrated 71% growth inhibition of *C. coccodes* at 100 μM, while **2** and **3** showed no significant growth inhibition.

A second bioassay revealed a moderate antioxidant activity for **1**, **2**, and **3** of 15%, 12%, and 21%, respectively, at 1 mM using the DPPH free radical scavenging method.

Experimental Section

General Experimental Procedures. 1D (^{1}H, ^{13}C) and 2D (HSQC, HMBC, COSY, ROESY) NMR spectra were recorded on a Varian Unity 500 NMR spectrometer operating at 499.81 and 125.01 MHz using a 3 mm microsample inverse detection probe and on a Bruker Avance 700 NMR spectrometer operating at 700.16 and 176.08 MHz equipped with an inverse cryoprobe. Chemical shifts were referenced to internal TMS (δ = 0 ppm, ^{1}H) or DMSO-d_6 (δ = 39.5 ppm, ^{13}C). Preparative HPLC was performed on a Varian ProStar 218 system with a Prep Star 330 photodiode array detector using an ODS C_{18} column (5 μm, 150 × 20 mm i.d., YMC). The ESI and collision-induced dissociation (CID) mass spectra were obtained from a TSQ Quantum Ultra AM system (Thermofinnigan) equipped with a hot ESI source (HESI, electrospray voltage: 3.0 kV, sheath gas: nitrogen; vaporizer temperature: 50 °C; capillary temperature: 250 °C; collision gas: argon; collision pressure: 1.5 mTorr). The MS system was coupled with a Surveyor Plus micro-HPLC (Thermo Electron) and equipped with an Ultrasep ES RP18E column (5 μm, 100 × 1 mm i.d., SepServ). For the HPLC, a gradient system was used starting from H_2O/CH_3CN (90:10) (each of them containing 0.2% HOAc) to 10:90 within 15 min; flow rate 50 μL min^{-1}. The high-resolution ESI mass spectra were obtained from a Bruker Apex III Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (Bruker Daltonics) equipped with an Infinity cell, a 7.0 T superconducting magnet (Bruker), a rf-only hexapole ion guide, and an external APOLLO electrospray ion source (Agilent, off-axis spray). The sample solutions were introduced continuously via a syringe pump with a flow rate of 120 μL h^{-1}. IR spectra were measured on a Thermo Nicolet 5700 FT-IR on an ATR crystal (diamond), and UV spectra on a Jasco V-560 UV/vis spectrometer. Column chromatography was performed on Diaion HP 20 (Suppelco), Sephadex LH-20 (Pharmacia), and silica gel RP-2 (Merck). The mycelium growth inhibition assay was carried out in 96-well microplates, and the absorbance was measured with a MRX plate reader 1.12 (Dynatech Laboratories) at 590 nm. The antioxidant activity was evaluated at a wavelength of 520 nm using a Genios Pro microplate reader (Tecan). Fluorescence spectra were obtained from a Perkin-Elmer MPF-44 spectrometer.

Fungal Material. *C. subtortus* (Pers.) Fr. was collected in Freudenstadt (Schwarzwald), Germany (October 3, 2006, leg./det. N. Arnold, coll. 37/06). Voucher specimens are deposited at the Leibniz Institute of Plant Biochemistry Halle, Germany (IPB).

Extraction and Isolation. Frozen fruiting bodies of *C. subtortus* (560 g) were crushed in a mixer and successively extracted with 2 L of MeOH at room temperature. The red-brown solution was concentrated to dryness *in vacuo* to a brown residue (12 g). The crude extract was adsorbed on Diaion HP-20 resin and fractionated by sequential elution with H_2O and MeOH. The MeOH fractions containing the fluorescent compounds **1**−**3** were further purified by repeated column chromatography on silica gel RP-2 using 50% aqueous MeOH and Sephadex LH-20 using 70% aqueous MeOH as solvent. Preparative HPLC on an ODS C-18 column using H_2O (A) and CH_3CN (B) as eluents (linear gradient: 0−20 min, 3−50% B, flow rate of 20.0 mL/min) afforded compounds **1** (2.0 mg), **2** (0.8 mg), and **3** (0.9 mg).

6-Hydroxyquinoline-8-carboxylic acid (1): yellow needles; mp 191−193 °C; UV (MeOH) λ_{max} (log ε) 214 (4.3), 233 (4.4), 298 (3.6), 347 (3.7) nm; IR ν_{max} 3282 (br), 2919, 2851, 1681, 1575, 1536, 1374, 1351, 1236,1205, 787 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 10.71 (1H, br s, O*H*), 8.87 (1H, dd, J = 4.4, 1.6 Hz, H-2), 8.54 (1H, dd, J = 8.5, 1.6 Hz, H-4), 8.13 (1H, d, J = 2.8 Hz, H-7), 7.71 (1H, dd, J = 8.5, 4.4 Hz, H-3), 7.54 (1H, d, J = 2.8 Hz, H-5); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 166.2 (C, C-9), 155.7 (C, C-6), 146.1 (CH, C-2), 139.5 (C, C-8a), 137.5 (CH, C-4), 129.9 (C, C-8), 125.8 (CH, C-3), 125.5 (C, C-4a), 122.4 (CH, C-7), 114.0 (CH, C-5); LC-ESI-CIDMS (positive ion mode, −15 eV) *m/z* (rel int, %) 190 ($[M + H]^+$, 33), 172 (100), 144 (4), 116 (3); ESI-FTICR-MS *m/z* 212.0319 ($[M + Na]^+$, calcd for $C_{10}H_7NO_3Na^+$, 212.0318).

4-Amino-6-hydroxyquinoline-8-carboxylic acid (2): white, amorphous residue; UV (MeOH) λ_{max} (log ε) 214 (2.4), 248 (2.3), 297 (0.9), 309 (1.0), 357 (1.9), 371 (1.9) nm; IR ν_{max} 2924 (br), 2853, 2160, 2026, 1976, 1726, 1580, 1377, 1244 cm^{-1}; ^{1}H NMR (DMSO-d_6, 700 MHz) δ 10.25 (1H, br s, O*H*), 8.35 (1H, d, J = 6.3 Hz, H-2), 8.08 (1H, d, J = 2.6 Hz, H-7), 8.00 (2H, br s, N*H*$_2$), 7.65 (1H, d, J = 2.6 Hz, H-5), 6.64 (1H, d, J = 6.3 Hz, H-3); ^{13}C NMR (DMSO-d_6, chemical shifts from HSQC and HMBC correlation peaks) δ 166.8 (C, C-9), 156.2 (C, C-6), 155.1 (C, C-4), 140.8 (CH, C-2), 136.9 (C, C-8a), 128.7 (C, C-8), 125.4 (CH, C-7), 119.3 (C, C-4a), 107.9 (CH, C-5), 100.8 (CH, C-3); LC-ESI-CIDMS (positive ion mode, −20 eV) *m/z* (rel int, %) 205 ($[M + H]^+$, 8), 187 (100), 159 (10), 132 (2), 131 (4); ESI-FTICR-MS *m/z* 227.0425 ($[M + Na]^+$, calcd for $C_{10}H_8N_2O_3Na^+$, 227.0427).

7-Hydroxy-1-oxo-1,2-dihydroisoquinoline-5-carboxylic acid (3): light brownish amorphous residue; UV (MeOH) λ_{max} (log ε) 212 (3.3), 246 (3.2), 300 (2.4), 354 (2.8), 365 (2.8) nm; IR ν_{max} 3358 (br), 3219 (br), 2922, 2851, 1650, 1570, 1355, 1210, 814 cm^{-1}; ^{1}H NMR (DMSO-d_6, 700 MHz) δ 14.88 ppm (1H, d, J = 5.6 Hz, N*H*), 9.38 (1H, br s, O*H*), 7.85 (1H, dd, J = 7.2, 5.6 Hz, H-3), 7.75 (1H, d, J = 3.0 Hz, H-6), 7.38 (1H, d, J = 3.0 Hz, H-8), 5.85 (1H, d, J = 7.2 Hz, H-4); ^{13}C NMR (DMSO-d_6, chemical shifts from HSQC and HMBC correlation peaks) δ 177.2 (C, C-1), 168.0 (C, C-9), 153.8 (C, C-7), 138.1 (CH, C-3), 135.7 (C, C-4a), 128.4 (C, C-5), 127.2 (C, C-8a), 123.2 (CH, C-6), 111.4 (CH, C-8), 107.0 (CH, C-4); LC-ESI-CIDMS

(negative ion mode, +20 eV) *m/z* (rel int, %) 204 ([M − H]$^-$, 11), 160 (100), 159 (37), 132 (6), 131 (3); ESI-FTICR-MS *m/z* 204.0299, [M − H]$^-$, calcd for $C_{10}H_6NO_4^-$, 204.0302).

Bioassays. *Colletotrichum coccodes* (Wallr.) S. Hughes was grown for 2 days in a soy-nutrient medium according to a reported composition.[26] The spores were subsequently transferred into fresh soy-nutrient medium (10^5 spores × mL^{-1}), and 200 μL of that suspension was used per well in the assay. After 1 day of sporulation, 20 μL of a solution of the test compounds was added to each well in final concentrations ranging from 10^{-9} to 10^{-3} M. The absorbance of the inoculate was measured at 590 nm every 24 h until a growth plateau of the fungus was detected. After six days of incubation the mycelium growth inhibition of each compound was determined. 8-Hydroxyquinoline sulfate (Sigma) was used as a positive control (EC_{50} = 0.19 μM).

Estimation of the radical scavenging activity was carried out using a DPPH free radical scavenger assay in 96-well microtiter plates according to a modified literature method.[27,28] Ascorbic acid (Sigma) was used as a positive control (EC_{40} = 45 μM).

Acknowledgment. The authors are indebted to Mrs. C. Kuhnt for LC-MS/MS, Mrs. M. Süsse for IR, and Dr. M. Findeisen for recording NMR spectra on a 700 MHz spectrometer, as well as Mr. L. Eschen-Lippold for support in the performance of the antifungal assay.

References and Notes

(1) http://www.indexfungorum.org/Names/Names.asp.
(2) Moser, M. In *Kleine Kryptogamenflora*; Gams, H., Ed.; Gustav Fischer Verlag: Stuttgart, 1983; pp 347−415..
(3) Garnica, S.; Weiss, M.; Oertel, B.; Oberwinkler, F. *Can. J. Bot.* **2005**, *83*, 1457–1477.
(4) Brandrud, T. E.; Lindström, H.; Marklund. H.; Melot, J.; Muskos, S. *Cortinarius, Flora Photographica*; Cortinarius HB: Matfors, Vols. 1 (1990), 2 (1992), 3 (1995), 4 (1998).
(5) Steglich, W.; Kopanski, L.; Wolf, M.; Moser, M.; Tegtmeyer, G. *Tetrahedron Lett.* **1984**, *25*, 2341–2344.
(6) Brondz, I.; Hoiland, K.; Bell, D. S.; Annino, A. R. *The Reporter* **2006**, *24*, 6–7.
(7) Erickson, E. (Riker Laboratories, Inc.) U.S. Patent 4,147, 694, 1979.
(8) Riker Laboratories, Inc., AustrianPatent AT 78,2821, 1978.
(9) Michael, J. P. *Nat. Prod. Rep.* **2003**, *20*, 476–493.
(10) Matsushima, H.; Fukumi, H.; Arima, K. *Agr. Biol. Chem.* **1973**, *37*, 1865–1871.
(11) Torigoe, K. *Vitamins* **1955**, *9*, 469–472.
(12) Stone, T. W. *Trends Pharmacol. Sci.* **2000**, *21*, 149–154.
(13) Mendez, J.; Masa, A. *Phytochemistry* **1975**, *14*, 1136–1137.
(14) Macnicol, P. K. *Biochem. J.* **1968**, *107*, 473–479.
(15) Starrat, A. N. *Phytochemistry* **1996**, *42*, 1477–1478.
(16) Al-Khalil, S. *J. Nat. Prod.* **1998**, *21*, 262–263.
(17) Nawwar, M. A. M.; Barakat, H. H.; Buddrus, J.; Linscheid, M. *Phytochemistry* **1985**, *24*, 818–819.
(18) Breiding-Mack, S.; Zeeck, A. *J. Antibiot.* **1987**, *40*, 953–960.
(19) Mossialos, D.; Meyer, J.-M.; Budzikiewicz, H.; Wolff, U.; Koedam, N.; Baysse, C.; Anjaiah, V.; Cornelis, P. *Appl. Environ. Microbiol.* **2000**, *66*, 487–497.
(20) Liu, J.-K. *Chem. Rev.* **2005**, *105*, 2723–2744.
(21) Abraham, W. R.; Spassov, G. *Phytochemistry* **1991**, *30*, 371–372.
(22) Pinard, E.; Alanine, A.; Bourson, A.; Büttelmann, B.; Heitz, M.-P.; Mutel, V.; Gill, R.; Trube, G.; Wyler, R. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2615–2619.
(23) Mackenzie, A. H. *Am. J. Med.* **1983**, *75*, 5–10.
(24) Chung, H. S.; Woo, W. S. *J. Nat. Prod.* **2001**, *64*, 1579–1580.
(25) Lees, A. K.; Hilton, A. J. *Plant Pathol.* **2003**, *52*, 3–12.
(26) Yu, X.; Hallett, S. G.; Sheppard, J.; Watson, A. K. *Appl. Microbiol. Biotechnol.* **1997**, *47*, 301–305.
(27) Blois, M. S. *Nature* **1958**, *181*, 1199–1200.
(28) Mohamad, H.; Abas, F.; Permana, D.; Lajis, N. H.; Ali, A. M.; Sukari, M. A.; Hin, T. T. Y.; Kikuzaki, H.; Nakatani, N. *Z. Naturforsch.* **2004**, *59 C*, 811–815.

NP8000859