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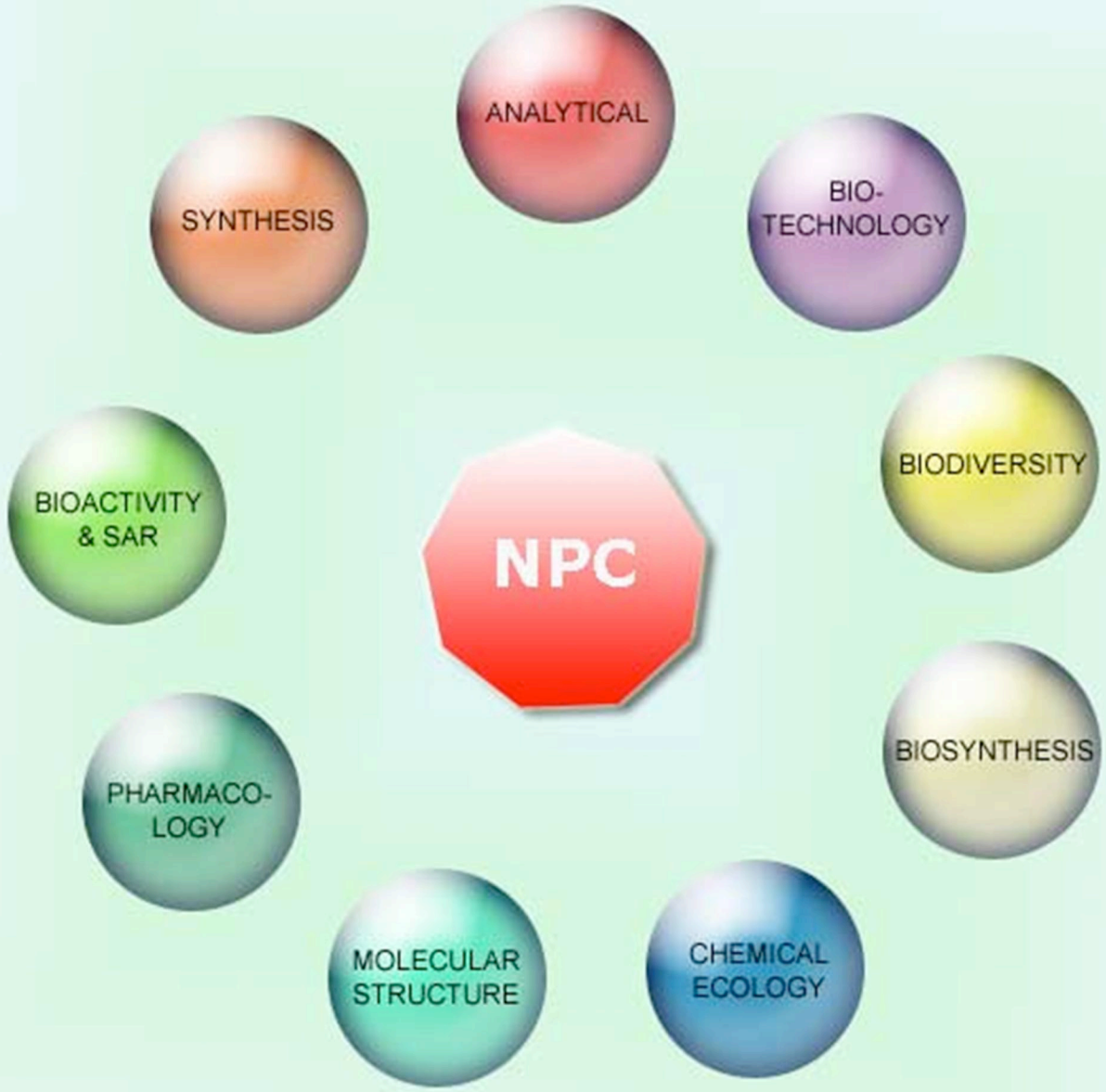

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Bioactive Isocoumarins from a Terrestrial *Streptomyces* sp. ANK302

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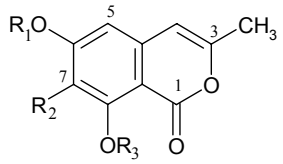
Four isocoumarins have been isolated from the terrestrial *Streptomyces* sp. ANK302, namely 6,8-dimethoxy-3-methylisocoumarin (**1**), 6,8-dihydroxy-3-methylisocoumarin (**2**), 6,8-dihydroxy-7-methoxy-3-methylisocoumarin (**3**), and 6,7,8-trimethoxy-3-methylisocoumarin (**4**). Compound **1** is a new naturally-occurring isocoumarin, and **2** was isolated as a new bacterial product. The structures **1-4** were deduced from high resolution mass, 1D and 2D NMR spectra and by comparison with related compounds from the literature. Compound **2** showed a strong zoosporicidal activity at a concentration of 5 µg/mL against a phytopathogenic oomycete, *Plasmopara viticola*, and **1** was active against *Candida albicans*.

Keywords: terrestrial *Streptomyces*, isocoumarins, zoosporicide, oomycetes.

Isocoumarins are not rare in plants and microorganisms and are forming an important class of compounds due to their broad biological activities, such as antimicrobial, antimalarial [1], antituberculous, cytotoxic, antifungal [2], immunomodulatory, and anti-inflammatory properties [3]. Isocoumarins are useful intermediates in the synthesis of a variety of natural products, including some isoquinoline alkaloids [4].

In plants, the number of coumarins is approximately three times higher than that of isocoumarins, but oppositely, in microorganisms, the latter group occurs three times more often [5-7].

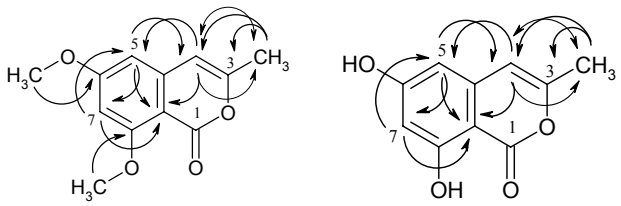
During our investigation of the terrestrial *Streptomyces* sp. ANK302, four isocoumarins (**1-4**) were isolated. Compounds **2-4**, including several related glycosides [8], had been described previously, but NMR data were not published. Interpretation of the HSQC and HMBC correlations (Figure 1), the molecular weights and corresponding formulas (by HRMS) unambiguously confirmed their structures and led to full assignment of their shift values. Compound **2** had been isolated previously from statically grown cultures of the fungus



1: $R_1 = CH_3$, $R_2 = H$, $R_3 = CH_3$
2: $R_1 = R_2 = R_3 = H$
3: $R_1 = H$, $R_2 = OCH_3$, $R_3 = H$
4: $R_1 = CH_3$, $R_2 = OCH_3$, $R_3 = CH_3$

Ceratocystis minor [9], the marine fungus *Keissleriella* sp. YS4108, and other fungi [10], while **3** and **4** have been isolated from an unidentified *Streptomyces* sp. [9]. 6,8-Dimethoxy-3-methylisocoumarin (**1**) is a new natural product.

6,8-Dimethoxy-3-methylisocoumarin (**1**) was obtained as UV absorbing colorless solid of medium polarity, which exhibited a red color reaction with anisaldehyde/H_2SO_4 spraying reagent. A molecular weight of 220 Daltons was determined by EI MS, and the (+)-HRESI MS confirmed the molecular formula as $C_{12}H_{12}O_4$. The ^{1}H NMR spectrum of **1** displayed doublets (J = 2.3 Hz) of two *m*-coupled aromatic protons at δ 6.41 and 6.29; a further 1H singlet



1 **2**

Figure 1: Selected H,H COSY (↔) and HMBC (→) correlations of isocoumarins **1** and **2**.

appeared at δ 6.08. In the aliphatic region, two methoxysinglets at δ 3.95 and 3.88 and a resonance of an sp^2-bound methyl singlet (δ 2.21) were visible.

A combination of ^{13}C and HSQC NMR spectra revealed all 12 carbon resonances of **1**, four at chemical shifts indicative of oxygenated quaternary carbons in the aromatic region (δ 165.2, 163.1, 159.4, and 155.3), two quaternary carbons at δ 142.3, 102.7, and three methines at δ 103.6, 99.3 and 98.0. Of the remaining resonances, two could be attributed to methoxy groups at δ 56.2 and 55.6, and a methyl signal appeared at δ 19.5.

Structure **1** was further confirmed by HMBC correlations (Figure 1): H-7 (δ 6.41) exhibited a ^3J correlation with C-8a (δ 102.7) and CH-5 (δ 99.3) in the tetrasubstituted aromatic ring (Figure 1). The methine doublet of H-5 exhibited ^3J couplings with the quaternary carbon atom C-8a and the methine carbon CH-7. H-5 (δ 6.29) displayed no COSY correlation with H-4, but showed a ^3J coupling to CH-4 (δ 103.6) and *vice versa*, confirming a *syn-peri*-position for both protons. The methyl group 3-CH_3 showed a ^4J COSY correlation with H-4, in addition to its ^2J and ^3J HMBC correlations with C-3 and C-4, respectively. This resulted in structure **1**.

Crude extracts of *Streptomyces* sp. ANK302 and isolated compounds were tested for their effects on the motility behavior of the zoospores of the grapevine downy mildew pathogen, *Plasmopara viticola* [11]. The bioassay revealed that both the crude extract (100 μg/mL) and compound **2** (5 μg/mL) inhibited motility of *P. viticola* zoospores in a dose- and time-dependent manner. Zoospores became paralyzed (100 %) and then immobilized (100%) within 60 min in the presence of compound **2** at the dose of 5 μg/mL. Compound **4** (25 μg/mL) inhibited the motility of *P. viticola* zoospores in a dose- and time-dependent manner as well. Zoospores became paralyzed (ca. 98 %) and then immobilized (ca. 73%) within 60 min. This is the first report on the motility inhibitory and zoosporicidal effects of isocoumarins against the infecting propagules of an important oomycete phytopathogen, which is insensitive to most of the chemical fungicides [12]. Compound **1** also showed antifungal activity against *Candida albicans* by causing an inhibition zone of 12 mm at 40 μg/disk. The other compounds did not show activities in our tests.

Experimental

General: UV/vis spectra were recorded on a Varian Cary 3E UV/vis spectrometer. NMR spectra were measured on Bruker AMX 300 (300.135 MHz), Varian Unity 300 (300.145 MHz) and Varian Inova 500 (499.876 MHz) spectrometers. EIMS were recorded on a Finnigan MAT 95 (70 eV). HRMS were recorded by ESI MS on an Apex IV 7 Tesla Fourier-Transform Ion Cyclotron Resonance Mass Spectrometer (Bruker Daltonics, Billerica, MA, USA). Flash chromatography was carried out on silica gel (230-400 mesh). Thin layer chromatography (TLC) was performed on Polygram SIL G/UV_{254} (Macherey-Nagel & Co.). R_f values were measured on Polygram SIL G/UV_{254} (Macherey-Nagel & Co.) with CH_2Cl_2/5% MeOH. Size exclusion chromatography was carried out on Sephadex LH-20 (Lipophilic Sephadex, Amersham Biosciences Ltd.; purchased from Sigma-Aldrich Chemie, Steinheim, Germany). XAD-16 resin was obtained from Rohm and Haas, France.

Isolation and taxonomy of Streptomyces sp. ANK302: The *Streptomyces* strain ANK302 has been derived from a soil sample and was isolated on YMG agar at room temperature (YMG agar: 2 g/L yeast extract, 5 g/L malt extract, 5 g/L glucose, 15 g/L agar, 30 mg/L cycloheximide). Its almost complete 16S rRNA gene sequence (GenBank Accession Nr. HM215581) shows high similarities to *Streptomyces rectiverticillatus* strain NRRL B-12369 (GenBank Accession Nr. DQ026657) and *S. aureoversilis* strain NBRC 13021 (GenBank Accession Nr. AB184855). The strain is deposited in the culture collection at the Institute of Organic and Biomolecular Chemistry, Göttingen, Germany.

Fermentation: The terrestrial isolate *Streptomyces* sp. ANK302 was inoculated on M_2 agar [13] from a soil storage culture and incubated for 96 h at 28°C. Three well-grown Petri plates were used to inoculate 100 of 1 L Erlenmeyer flasks, each containing 250 mL of M_2 medium, which were incubated as shake-cultures (95 rpm) at 28°C for 7 days. The resulting gray culture broth was mixed with *ca*. 1 kg diatomaceous earth (Celite) and pressed through a pressure filter affording the aqueous filtrate and the mycelial residue. The aqueous fraction was extracted with Amberlite XAD-16 resin and eluted using MeOH. The mycelium was extracted (3×) with EtOAc followed by acetone (1×). The acetone was evaporated and the aqueous residue extracted with EtOAc. Both organic phases were combined and evaporated to dryness, yielding 4.0 g of a brown extract, which was dissolved in methanol and extracted with cyclohexane to remove fats. Flash chromatography on silica gel with a MeOH/CH_2Cl_2 gradient (column 3 × 60 cm, 0 to 20 % MeOH) afforded 3 fractions. Fractions 1 and 2 were further purified on Sephadex LH-20 (MeOH) to deliver compounds **2** (12 mg) and **3** (6 mg), respectively, while fraction 3 was separated on Sephadex LH-20 (MeOH), followed by silica gel (cyclohexane/EtOAc gradient 0 to 100 % EtOAc), and

again on Sephadex LH-20 (MeOH) to afford compounds **1** (5 mg) and **4** (10 mg).

6,8-Dimethoxy-3-methylisocoumarin (**1**)

Colorless solid.

R_f: 0.53 (CH_2Cl_2/5% MeOH)

UV λ_{max} (MeOH) nm (log ε): 324 (3.80), 277 (3.91); λ_{max} (MeOH/HCl) nm (log ε): 325 (3.72), 277 (3.84); λ_{max} (MeOH/NaOH) nm (log ε): 322 (3.75), 278 (3.84).

^{1}H NMR ($CDCl_3$, 300 MHz): δ 6.41 (1 H, d, J = 2.3 Hz, H-7), 6.29 (1 H, J = 2.3 Hz, H-5), 6.08 (1H, s, H-4), 3.95 (3H, s, 8-OCH_3), 3.88 (3 H, s, 6-OCH_3), 2.21 (3 H, s, 3-CH_3).

^{13}C NMR ($CDCl_3$, 125 MHz): δ 165.2 (C_q-6), 163.1 (C_q-8), 159.4 (CO-1), 155.3 (C_q-3), 142.3 (C_q-4a), 103.6 (CH-4), 102.7 (C_q-8a), 99.3 (CH-5), 98.0 (CH-7), 56.2 (8-OCH_3), 55.6 (6-OCH_3), 19.5 (3-CH_3).

EIMS (70 eV): *m/z* (%) 220 $[M]^{+\cdot}$ (100), 191 (66), 149 (64), 43 (34); ESI HRMS *m/z*: $[M+H]^+$ calcd for $C_{12}H_{13}O_4$: 221.080835; found: 221.0809070; $[M+Na]^+$ calcd for $C_{12}H_{12}O_4Na$: 243.06278; found: 243.06287.

6,8-Dihydroxy-3-methylisocoumarin (**2**)

Colorless solid.

R_f: 0.29 (CH_2Cl_2/5% MeOH)

^{1}H NMR (DMSO-d_6, 300 MHz): δ 10.95 (1H, brs, 8-OH), 6.43 (1 H, s, H-4), 6.31 (1 H, d, J = 2.1 Hz, H-5), 6.29 (1H, d, J = 2.1 Hz, H-7), 2.19 (3 H, s, 3-CH_3).

^{13}C NMR (DMSO-d_6, 125 MHz): δ 165.6 (CO-1), 165.2 (C_q-6), 162.5 (C_q-8), 153.8 (C_q-3), 139.4 (C_q-4a), 104.0 (CH-4), 102.3 (CH-5), 101.2 (CH-7), 97.0 (C_q-8a), 18.7 (CH_3).

EIMS (70 eV): *m/z* (%) 192 $[M]^{+\cdot}$ (100), 177 (50), 150 (16), 121 (20); ESI HRMS *m/z*: $[M+H]^+$ calcd for $C_{10}H_9O_4$: 193.04953; found: 193.04950; $[M+Na]^+$ calcd for $C_{10}H_8O_4Na$: 215.03148; found: 215.03146.

6,8-Dihydroxy-7-methoxy-3-methylisocoumarin (**3**)

Colorless solid.

R_f: 0.50 (CH_2Cl_2/5% MeOH).

^{1}H NMR (CD_3OD, 300 MHz): δ 6.38 (1 H, s, H-5), 6.30 (1H, s, H-4), 3.84 (3 H, s, 7-OCH_3), 2.19 (3 H, s, 3-CH_3).

^{13}C NMR (CD_3OD, 125 MHz): δ 168.2 (CO-1), 161.0 (C_q-6), 156.2 (C_q-8), 154.6 (C_q-3), 136.2 (C_q-4a), 135.5 (C_q-7), 105.3 (CH-4), 104.0 (CH-5), 100.0 (C_q-8a), 60.9 (7-OCH_3), 19.1 (3-CH_3).

EIMS (70 eV): *m/z* (%) 222 $[M]^{+\cdot}$ (100), 207 (90), 179 (60), 101 (38), 59 (44), 43 (34); ESI HRMS *m/z*: $[M+H]^+$ calcd for $C_{11}H_{11}O_5$: 223.060105; found: 223.0602940; $[M+Na]^+$ calcd for $C_{11}H_{10}O_5Na$: 245.042045; found: 245.0422940.

6,7,8-Trimethoxy-3-methylisocoumarin (**4**)

Colorless solid.

R_f: 0.55 (CH_2Cl_2/5% MeOH).

^{1}H NMR (CD_3OD, 300 MHz): δ 6.75 (1 H, s, H-5), 6.29 (1 H, s, H-4), 3.93 (3 H, s, 6-OCH_3), 3.88 (3 H, s, 8-OCH_3), 3.82 (3 H, s, 7-OCH_3), 2.20 (3 H, s, 3-CH_3).

^{13}C NMR (CD_3OD, 125 MHz): δ 161.6 (CO-1), 160.9 (C_q-6), 156.3 (C_q-8), 155.4 (C_q-3), 143.4 (C_q-7), 138.2 (C_q-4a), 107.7 (C_q-8a), 104.6 (CH-4), 104.0 (CH-5), 62.3 (8-OCH_3), 61.6 (7-OCH_3), 56.8 (6-OCH_3), 19.3 (CH_3-9).

EIMS (70 eV): *m/z* (%) 250 $[M]^{+\cdot}$ (56), 235 (100), 207 (40), 43 (36); ESI HRMS *m/z*: $[M+H]^+$ calcd for $C_{13}H_{15}O_5$: 251.09140; found: 251.09154; $[M+Na]^+$ calcd for $C_{13}H_{14}O_5Na$: 273.07334; found: 273.07350.

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[13] M_2 medium: Glucose (4 g), yeast extract (4 g), malt extract (10 g) were dissolved in 1 L of tap water. The pH of the solution was adjusted to 7.8 and sterilized by autoclaving for 30 minutes at 121°C and 1.2 bar.

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