Diastereomeric Quinolinone Alkaloids from the Marine-Derived Fungus *Penicillium janczewskii*[||]

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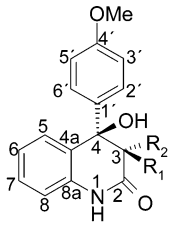
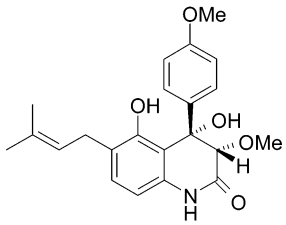
From *Penicillium janczewskii*, obtained from a marine sample, two new diastereomeric quinolinones, 3*S**,4*R**-dihydroxy-4-(4′-methoxyphenyl)-3,4-dihydro-2(1*H*)-quinolinone (**1**) and 3*R**,4*R**-dihydroxy-4-(4′-methoxyphenyl)-3,4-dihydro-2(1*H*)-quinolinone (**2**), were identified, along with two known alkaloids, peniprequinolone (**3**) and 3-methoxy-4-hydroxy-4-(4′-methoxyphenyl)-3,4-dihydro-2(1*H*)-quinolinone (**4**). Cytotoxicity testing on eight tumor cell lines revealed a moderate specificity of **2** on SKOV-3 cells.

The exploitation of the marine environment has been intriguingly successful in recent years in the search for structurally unusual and biologically highly active natural products.[1] To avoid depletion of marine resources and to enable access to large quantities of interesting compounds, there is a particular interest in those marine organisms that are culturable. Thus, we are studying microbial fungi isolated from marine sources for their potential of providing new natural products.[2] In a combined approach of biological and chemical screening[3] we are gaining a thorough understanding of the secondary metabolite pattern of these fungi.

Penicillium janczewskii Zalessky[4,5] (strain H-TW5/869) was selected from our screening program for further studies, because of a rich metabolite pattern as detected by thin-layer chromatography on silica gel with various staining reagents, and cytotoxic activity of its methanolic extracts. The extracts from mycelia and culture filtrate showed significant cytotoxicity against CHO-K1 cells. In addition to two known fungal metabolites (**3** and **4**), our studies yielded new diastereomeric dihydroquinoline alkaloids **1** and **2**. We here report the isolation and structure elucidation of **1** and **2**, as well as the biological activity of these compounds.



1 R_1 = H, R_2 = OH
2 R_1 = OH, R_2 = H
4 R_1 = OMe, R_2 = H



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The isolation of the metabolites was performed from a crude product of a 50 L cultivation of *P. janczewskii* H-TW5/869. Because of experimental feasibility and the predominant occurrence of metabolites, the culture filtrate was chosen for compound isolation. It was extracted through adsorption on Amberchrom CG161c resin and subsequent elution with MeOH. Initial separation of the natural products from the concentrated and lyophilized elute (14 g) was performed by flash reversed-phase chromatography (RP18, MeOH/H_2O gradient). The nonpolar fraction was repeatedly submitted to column chromatography ($CHCl_3$/MeOH) and finally purified by preparative HPLC (RP18, MeOH/H_2O gradient) to yield compounds **1** (2 mg) and **2** (3 mg).

Compound **1** was obtained as a white solid, and its molecular formula was established as $C_{16}H_{15}NO_4$ by HREIMS (*m/z* 285.0994 $[M]^+$, Δ0.7 mmu, 228.1040 $[M - C_2HO_2]^+$, Δ −1.5 mmu) and supported by ESIMS, which revealed the quasi-molecular weight through peaks at *m/z* 308.1 $[M + Na]^+$ and 593.2 $[2M + Na]^+$. The molecular formula indicated 10 degrees of unsaturation within the molecule. The IR bands at 1705, 1684, 1604, and 1511 cm^{-1} suggested the presence of a carbonyl group and an aromatic ring. ^{13}C NMR and DEPT data revealed one methoxy group (δ 54.9), two oxygenated sp^3-hybridized carbons [one methine (δ 74.8) and one quaternary carbon (δ 75.8)], 12 aromatic carbons, eight of which were methine groups, and one amide carbonyl carbon (δ 170.4). The ^1H NMR spectrum indicated that the aromatic signals represented two sets of protons: one was a pair of *ortho*-coupled doublets attributed to four symmetrical protons at δ 7.18, 6.78, and another was an ABCD system at δ 7.38, 7.22, 6.99, and 6.89, respectively. Furthermore, one methoxy group at δ 3.68 and one methine group at δ 4.29 were confirmed. Thus, it was determined that the basic skeleton of **1** consisted of two aromatic rings, one of them substituted with a cyclic amide. Cross-peaks in the HMBC spectrum of **1** between H-3 and C-2, C-4, C-4a and between NH-1 and C-2, C-3, C-4a, C-8, C-8a suggested the presence of a cyclic amide (Figure 1). HMBC correlations between OCH_3 (δ 3.68) and C-4′ (δ 158.2) revealed that the methoxy group was located at C-4′ of the benzyl ring, and those between H-3 and C-1′ and H-2′/6′ and C-4 revealed the location of that aromatic system. Finally, comparison of the ^1H and ^{13}C NMR spectral data of **1** with analogous data for the known peniprequinolone (**3**) and **4**,[6−8] which were also obtained

[||] Dedicated to Professor Dr. Axel Zeeck on the occasion of his 65th birthday.

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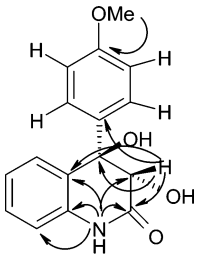

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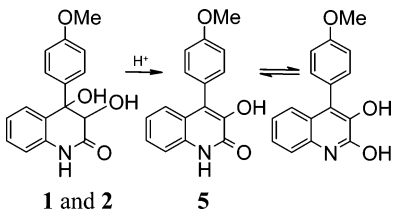
Figure 1. Key HMBC correlations of **1**.

Table 1. ^{13}C and ^{1}H NMR Data[a] for **1** and **2**

	1		**2**	
position	δ_C[b]	δ_H[c]	δ_C[b]	δ_H[c]
3	74.8	4.29 (s)	74.4	4.46 (s)
2	170.4		170.4	
4	75.8		76.5	
4a	131.1		129.5	
5	128.4	7.38 (dd, 1.0, 7.0)	128.7	6.74 (brd, 7.0)
6	122.5	6.99 (ddd, 1.0, 7.0, 8.0)	122.0	6.87 (m)
7	126.5	7.22 (dt, 1.0, 8.0)	128.0	7.21 (dt, 1.0, 7.0)
8	115.2	6.89 (dd, 1.0, 8.0)	115.3	6.90 (m)
8a	135.7		137.1	
1′	133.4		134.4	
2′, 6′	128.5	7.18 (d, 9.0)	128.1	7.28 (d, 9.0)
3′, 5′	112.6	6.78 (d, 9.0)	113.0	6.89 (d, 9.0)
4′	158.2		158.2	
1-NH		10.19 (brs)		10.46 (brs)
3-OH		5.30 (brs)		5.12 (brs)
4-OH		5.90 (brs)		5.48 (brs)
OCH_3	54.9	3.68 (s)	55.0	3.74 (s)

[a] In DMSO-d_6; chemical shifts in ppm. [b] 75 MHz. [c] 300 MHz; multiplicities and J values (Hz) are shown in parentheses.



1 and **2** **5**

Figure 2. Acidic dehydration of compounds **1** and **2**.

in the present investigation, indicated a family of structurally related compounds with differences in their substitution patterns. Thus, **1** was determined to be 3,4-dihydroxy-4-(4′-methoxyphenyl)-3,4-dihydro-2(1*H*)-quinolinone.

Compound **2** was isolated as a white amorphous solid. The molecular formula was also determined as $C_{16}H_{15}NO_4$ by HRESIMS of $[M + Na]^+$. The compound was recognized as a diastereomer of **1** with different relative stereochemistry at positions 3 and 4 on the basis of its very similar spectroscopic data (Table 1, Experimental Section). This assumption was proven through acidic dehydration by TsOH in acetone. A mixture of **1** and **2** (1:1) yielded 3-hydroxy-4-(4-methoxyphenyl)-2(1*H*)-quinolinone (**5**) as the sole product (Figure 2). The structure of **5** was unambigiously assigned through ^{1}H NMR and ESIMS data.[9,10]

The relative stereochemistry of **1** and **2** was determined through conformational analysis and quantitative NOE data obtained from NOESY spectra. Basic conformational analysis by different standard calculation methods (AM2, MMDO) clearly indicated an almost perpendicular orientation of the two aromatic rings with a significant rotational barrier around the C-4−C-1′ bond. A fixed conformational arrangement of the amide ring is rather unlikely. It can be assumed, though, that such conformational states in which H-3 and H-2′/6′ are situated in close proximity are more likely in the *syn*-facial arrangement of compound **2** than in the *anti*-facial situation of compound **1**. The observed NOE correlations between H-2′/6′ and H-3 were quantified by peak volumes and calibrated against the vicinal aromatic NOE between H-6 and H-7 (Figure 1). These calculations yielded a 13-fold higher peak volume of the NOE between H-3 and H-2′/6′ in compound **2** compared to that in compound **1**. Therefore, the structure of compound **1** was determined as 3*S**,4*R**-dihydroxy-4-(4′-methoxyphenyl)-3,4-dihydro-2(1*H*)-quinolinone, and **2** was 3*R**,4*R**-dihydroxy-4-(4′-methoxyphenyl)-3,4-dihydro-2(1*H*)-quinolinone. The diastereomers are very likely biosynthetic hydration products of an intermediate related to compound **5**.

Table 2. Cytotoxicity Data[a] of Compounds **1**−**3**

cell line	**1**	**2**	**3**
MDA-MB 231	52.9	31.8	8.4
DU-145	50.6	48.4	30.8
SKOV-3	44.6	8.1	20.2
HT-29	58.8	32.8	4.0
A549	56.3	56.8	nd
CAKI-1	80.3	65.1	nd
SK-MEL 2	55.2	39.0	nd
K562	52.4	36.6	nd

[a] % viability at 10 μg/mL compound concentration; nd not determined.

Peniprequinolone (**3**)[6] and 3-methoxy-4-hydroxy-4-(4′-methoxyphenyl)-3,4-dihydro-2(1*H*)-quinolinone (**4**)[7,8] are two known quinolinone type alkaloids that in this study were for the first time isolated from a marine-derived fungus. These compounds were originally isolated by bioassay-guided methods from the fungus *P.* cf. *simplicissimum* with nematicidal activity toward *Pratylenchus penetrans* of **3** and toxicity against brine shrimp of **4**, respectively. Compounds **1**−**4** obtained in this study were evaluated for their cytotoxic potential against human tumor cell lines (Table 2) by determining cell viability at a standard concentration of 10 μg/mL. The two new compounds **1** and **2** showed a low to moderate general toxicity against MDA-MB 231 (human breast adenocarcinoma), DU-145 (human prostate carcinoma), HT-29 (human colon carcinoma), A549 (human non small cell lung carcinoma), CAKI-1 (human kidney carcinoma), SK-MEL 2 (human melanoma), and K562 (human myeloid leukemia) cells, with **2** being slightly more potent. In addition, a significantly stronger cytotoxicity against SKOV-3 cells (human ovary adenocarcinoma) was found for **2**. As **1** and **2** differ only in their relative stereochemistry, these significant differences in efficacy as well as selectivity are very surprising. Finally, in accordance with its reported antifungal activity,[6] compound **3** acts as a strong cytotoxic agent, however, with no selectivity; **4** did not show significant cytotoxicity.

Experimental Section

General Experimental Procedures. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter. IR spectra were recorded with a Perkin-Elmer model 298 spectrometer (KBr, disks). 1D NMR (^{1}H, ^{13}C) spectra were recorded on a Bruker DPX-300 spectrometer; 2D NMR (COSY, HMBC, HMQC, NOESY) spectra, on a Bruker DPX-500 spectrometer. HREIMS were recorded with the AMD-402 instrument of Be geometry equipped with direct inlet system (AMD Intectra). ESIMS were recorded by use of a Quattro triple quadrupole mass spectrometer (VG Biotech, Altrincham, England) and Finnigan MAT 311A (EI: 70 eV, direct inlet, high resolution with perfluorokerosine as a standard). TLC was performed on silica gel plates (Macherey-Nagel, Sil G/UV_{254}, 0.20 mm); spots were detected under a UV lamp and after staining with

anisaldehyde/H_2SO_4. Solid-phase extraction was done on Amberchrom CG-161C (TOSOHAAS); TLC was performed on DC Alugram SIL G/UV 254 (0,25 mm, Macherey-Nagel GmbH&Co, Germany), and staining reagents were prepared according to standard laboratory procedures. Staining was achieved through spraying onto the plate and subsequent heating. Chromatography was done on LiChroprep RP-18 (25−40 μm, Merck KGaA, Germany), Sephadex LH-20 (Pharmacia Biotech AB, Sweden), and silica gel 60 (0.040−0.063 mm, Merck KgaA, Germany). All solvents were of gas-distilled grade.

Microorganism and Fermentation. The strain of *Penicillium janczewskii* Zalessky (strain KMPB H-TW5/869) was isolated from surface water collected during a research cruise in 1995 from the German Bight (North Sea) northeast of the island Helgoland (54°22′0″ N, 8°16′0″ E). The fungus was maintained on agar slants before the identification according to the protocol of Pitt.[4,5] The isolate was deposited as DSM17433 at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). Inocculum was prepared from agar slants. After 7 days of cultivation at 25 °C spores and mycelium were cut by a cork borer and used for inocculating the culture broth. The large-scale fermentation was performed in resting cultures in Fernbach flasks in a total volume of 50 L in glucose/potato extract broth.[4] Screening cultures were run in 200 mL of culture media in Erlenmeyer flasks. The cultures were incubated at 25 °C for 29 days.

Screening Extracts. Cultures were separated by filtration. The lyophilized mycelium was extracted with 50 mL of methanol. The suspension was sonicated for 6 min, and extraction was performed for 3 h. The solvent of the separated extract was removed in vacuo, and the residue was dissolved in 1 mL of methanol and stored at −20 °C in microplates. Samples of the culture filtrate were prepared by solid-phase extraction on Amberchrom 161c (Supelco).[11]

Downstream Processing and Purification. After upscale cultivation, biomass and culture liquid were separated by filtration through cheesecloths. Biomass was lyophilized to give 312 g of dried mycelia. The culture broth was adsorbed on a Amberchrom CG 161c column, washed with distilled water, and eluted with methanol. The methanolic extract was evaporated in vacuo to give 14 g of crude extract. The lyophilized culture filtrate was separated by flash chromatography (RP18, MeOH/H_2O, gradient) to obtain fractions 1 to 4. Fraction 4 was purified using Sephadex LH-20 (3 × 80 cm, MeOH) combined with repeated column chromatography ($CHCl_3$/MeOH, gradient) on silica gel to yield compound **3** (15 mg), compound **4** (20 mg), and a mixture of **1** and **2** The latter was further purified by preparative HPLC (RP18, MeOH/H_2O gradient from 25/75 to 45/55 in 110 min) to obtain **1** (2 mg, $t_{R,1}$ = 45 min) and **2** (3 mg, $t_{R,2}$ = 55 min).

Cytotoxicity Testing. The cytotoxic activities of methanolic extracts and pure compounds were determined in a sulforhodamine B (SRB) staining assay (Sigma, Schnelldorf, Germany). For a typical experiment, cells were inoculated in 96-well microtiter plates in amounts of 90 μL at plating densities ranging from 5000 to 10 000 cells/well depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated for 24 h prior to addition of the screening extracts or compounds. Extracts (50% MeOH) or compounds solubilized in 100% MeOH or dimethyl sulfoxide were stored frozen prior to use. The final test concentration for pure compounds of 1 μg/test well (100 μL) with complete medium corresponds to a micromolar test range. Following extract or compound addition, the plates were incubated for an additional 48 h, then washed with PBS. The assay was terminated by fixing of the cells with cold trichloroacetic acid solution (TCA) for 30 min on ice (final concentration: adherent cells 10% TCA; suspension cells 16% TCA). The supernatant was discarded, and the plates were washed five times with tap water and air-dried. SRB solution (100 μL) at 0.4% (w/v) in 1% acetic acid was added to each well, and plates were incubated for 10 min at room temperature. After staining, unbound dye was removed by washing five times with 1% acetic acid and the plates were air-dried. Bound stain was subsequently solubilized in 10 mM Tris/KOH (pH 10.5), and the absorbance was read at 550 nm.

3*S,4*R**-Dihydroxy-4-(4′-methoxyphenyl)-3,4-dihydro-2(1*H*)-quinolinone (1):** white solid; $[\alpha]^{15}_D$ −12.9° (*c* 0.7, MeOH); IR (KBr) ν_{max} 3300, 1705, 1684, 1604, 1511 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HMBC correlations (DMSO-d_6) NH-1/C-2, NH-1/C-3, NH-1/C-4a, NH-1/C-8, NH-1/C-8a, H-3/C-2, H-3/C-4, H-3/C-4a, H-3/C-1′, OH-3/C-1′, OH-3/C-3, OH-3/C-4, OH-4/C-3, OH-4/C-4, OH-4/C-4a, OH-4/C-1′, H-5/C-4, H-5/C-8a, H-6/C-5, H-6/C-7, H-6/C-8, H-6/C-4a, H-6/C-8a, H-7/C-6, H-7/C-8a, H-7/C-8, H-8/C-4, H-8/C-6, H-8/C-4a, H-8/C-8a, H2′(6′)/C-4, H2′(6′)/C-3′(5′), H2′(6′)/C-4′, H3′(5′)/C-1′, H3′(5′)/C-4′, OCH_3/C-4′; ESIMS *m/z* 308.1 $[M + Na]^+$, 285.9 $[M + H]^+$, 593.2 $[2M + Na]^+$.

3*R,4*R**-Dihydroxy-4-(4′-methoxyphenyl)-3,4-dihydro-2(1*H*)-quinolinone (2):** white solid; $[\alpha]^{15}_D$ −4.2° (*c* 0.5, MeOH); IR (KBr) ν_{max} 3200, 1710, 1678, 1612, 1511 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; ESIMS *m/z* 308.1 $(M + Na)^+$, 593.5 $[2M + Na]^+$; HRESIMS *m/z* 308.0911 $[M + Na]^+$ (calcd for $C_{16}H_{15}NO_4Na$, 308.0899).

Acidic Dehydration of 1 and 2. To a solution of **1** and **2** (1:1, 3 mg) in acetone (2 mL) TsOH was added (0.5 mg), and the mixture was stirred at room temperature for 2 h. After solvent evaporation, the residue was purified by silica gel column chromatography (P.E./EtOAc, 4:1) to give compound **5** (1 mg).

3-Hydroxy-4-(4′-methoxyphenyl)-2(1*H*)-quinolinone, 5: white solid; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.31 (1H, brs, NH), 7.33 (1H, d, *J* = 7.6 Hz), 7.25 (1H, m), 7.11 (2H, d, *J* = 9.0 Hz), 7.07 (1H, t, *J* = 7.4 Hz), 6.85 (1H, d, *J* = 7.9 Hz), 6.80 (2H, d, *J* = 9.0 Hz), 4.35 (1H, s, OH), 3.75 (3H, s, OCH_3); ESIMS *m/z* 268.1 [M + H].

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