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Isolation and Structure Elucidation of Two New Antibacterial Compounds Produced by *Chrysosporium queenslandicum*

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During our investigation on bioactive antifungal pigments the strain *Chrysosporium queenslandicum* IFM 51121 was found to produce a series of darkish to reddish pigments which were identified as naphthaquinone-type altersolanols A, B, C[1~3]. Moreover, a new antifungal compound (queenslandon)[4] was isolated as a new representative of the zearalenone family of mycotoxin. In addition to the above metabolites *C. queenslandicum* IFM 51121 co-produced new antibacterial compounds related to members of the dihydronaphthaquinone group[5~7] which were given the name chrysoqueen (**1**) and chrysolandol (**2**). In this paper we report fermentation, isolation, physico-chemical properties and the structure elucidation of **1** and **2**.

A culture sample of *C. queenslandicum* IFM 51121 was deposited in the microbial strain collection of the Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Japan.

The mature slant culture of *C. queenslandicum* IFM 51121 was inoculated into a 50 ml Erlenmeyer shake flask containing 25 ml of a seed medium (2% glucose supplemented BHI medium). The inoculated flasks were shaken at 250 rpm at 32°C for 3 days. The seed cultures (20 ml) were transferred to a 5 liter Erlenmeyer flask containing 2.5 liters of a medium (3% glucose supplemented PDB medium). After 25 days of cultivation at 36°C, the culture broth (7.5 liters) of *C. queenslandicum* IFM 51121 was centrifuged at 5°C for 30 minutes at 5,000 rpm. The mycelium was discarded and the supernatant fluid was run through a Diaion HP 20 column (5×15 cm). The column was washed with water and subsequently eluted with methanol - water (1 : 1 and 1 : 0). Fractions showing activity against *Bacillus subtilis* PCI 219, *Micrococcus luteus* IFM 2066 and *Aspergillus niger* IFM 5368 were pooled and concentrated *in vacuo*. The residue was suspended in water and extracted with ethyl acetate (600 ml). After washing of the ethyl acetate layer with water and drying over Na_2SO_4, the extract was evaporated to dryness under reduced pressure yielding 150 mg crude product. It was dissolved in chloroform and chromatographed on a silica gel FL 100D column equilibrated with chloroform, using a gradient of 20% to 90% methanol in chloroform. Fractions showing activity against *B. subtilis* PCI 219 and *Micrococcus luteus* IFM 2066 were combined. The solvent was evaporated *in vacuo*, the residue was dissolved in a small amount of ethyl acetate and further purification and separation was achieved by preparative thin-layer chromatography on silica gel plates (Merck silica gel 60 F_{254}) using mixtures of $CHCl_3$ - MeOH (95 : 5; v/v) and (80 : 20; v/v). The compounds were disclosed on the TLC plates due to their yellowish fluorescence in UV_{254} light. The zones of the naphthaquinone antibiotics altersolanols A, B, C and chrysoqueen (**1**) were eluted and rechromatographed on silica gel plates using $CHCl_3$ - MeOH - H_2O (14 : 3 : 0.1; v/v/v). Final purification of **2** occurred by preparative TLC on the same plates using $CHCl_3$ - MeOH (80 : 20; v/v), followed by rechromatography in $CHCl_3$ - MeOH (75 : 25; v/v). At the end of the purification procedure 20 mg of **1** and 25 mg of **2** were obtained. The physico-chemical properties of the chrysoqueen (**1**) and chrysolandol (**2**) are shown in Table 1.

In the UV spectra of **1** and **2** carbonyl groups in

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Table 1. Physico-chemical properties of **1** and **2**.

	1	**2**
Appearance	Slightly yellowish wax	yellowish wax
Molecular weight	382	354
Formula	$C_{17}H_{18}O_{10}$	$C_{16}H_{18}O_9$
EI-MS	m/z 338.1001 $[M-CO_2]^+$ found, calcd. 338.1007 for $C_{16}H_{18}O_8$	m/z 354.0967 $[M^+]$ found, calcd. 354.0951
UV-VIS (λ_{max}, nm, in MeOH)	214, 229, 287, 329 (shoulder)	210, 246, 285, 350
IR (λ_{max}, cm^{-1}) film (ATR)	780, 920, 950, 1020, 1107, 1159, 1377, 1455, 1595, 1625, 1739, 1842, 2850, 2919, 3325, 3335	776, 850, 938, 976, 1024, 1074, 1178, 1203, 1247, 1289, 1339, 1377, 1492, 1573, 1613, 1635, 1711, 2850, 2918, 3289
$[\alpha]_D^{22}$ (methanol)	+ 9.3 °	+ 36.0°
R_f (TLC) Chloroform-methanol (8:2)	0.75	0.38
HPLC (R_t, min) Gradient of 20% to 90% acetonitrile in water	15.59	14.44

neighbourhood to an aromatic system were visible (Table 1). The infrared spectra of **1** and **2** (FT-IR instrument, Mattson, USA) showed diagnostic absorbances of carbonyles, double bonds and hydroxyl groups. The molecular weights and the chemical formulas of **1** and **2** were furnished by mass spectrometry. The ESI-MS of **1** (Triple Quadrupole Instrument Quattro, VG Biotec, Altrincham, England) displayed in the positive ion mode *m/z* 405.0 ($[M+Na]^+$, 100%) and *m/z* 787.3 ($[2M+Na]^+$, 72%). In the negative ion mode *m/z* 381.3 ($[M-H]^-$, 100%) and *m/z* 763.5 ($[2M-H]^-$, 50%) were visible suggesting a molecular weight of 382 Da. In accord with this suggestion the HREI-MS (Finnigan MAT XL95, Bremen, Germany) showed *m/z* 338.1001 ($[M-CO_2]^+$, calcd. 338.1007 for $C_{16}H_{18}O_8$) suggesting $C_{17}H_{18}O_{10}$ as the chemical formula of **1** (Table 1). The ESI-MS of **2** displayed *m/z* 377.2 ($[M+Na]^+$, 100%), *m/z* 731.0 ($[2M+Na]^+$, 66%), *m/z* 353.2 ($[M-H]^-$, 100%) and *m/z* 707.0 ($[2M-H]^-$, 60%). HREI-MS of **2** afforded *m/z* 354.0967 (M^+, calcd. 354.0951 for $C_{16}H_{18}O_9$). The chemical formulas of **1** and **2** suggested the presence of nine and eight rings or double bonds, respectively. The structures of **1** and **2** (relative stereochemistry) as shown in Fig. 1 were assigned by 1D and 2D NMR measurements (^1H, ^{13}C, DEPT, COSY, HMQC, HMBC, NOESY). The ^1H-NMR spectrum of **1** showed two *meta*-coupled aromatic protons signals (6.62 ppm and 6.39 ppm), one pair of methylene protons (1.96 ppm; 2.12 ppm), three protons coupled oxygen-bonded carbons (4.65 ppm, 4.67 ppm, 5.29 ppm), one methyl group (1.30 ppm), one methoxyl group (3.80 ppm, s), and four hydroxyl protons appearing at 5.6 ppm (broad), 6.0 ppm, 7.01 ppm and 12.0 ppm. The downfield shift of the latter suggested its β-position relative to a carbonyl group.

In the ^{13}C NMR spectrum of **1** seventeen carbons were visible (Table 2) which were assignable to carbonyles (154.5 ppm, 198.2 ppm), six aromatic carbons, one methoxyl, one methyl, one methylene and six oxygen-bonded carbons. According to the DEPT spectrum the latter three were quaternary, oxygen-bonded carbons (74.2 ppm, 68.6 ppm, 78.2 ppm). The unusual upfield shift of the carbonyl C-13 (154.5) was readily explained by the presence of a cyclic carbonate structure[8].

The ^1H NMR spectrum of **2** showed two *meta*-coupled

Table 2. Assignments of ^{1}H and ^{13}C NMR data (500 MHz) of **1** and **2** (in DMSO, TMS as internal standard, chemical shifts in ppm) multiplicity in parentheses coupling constants Hz.

Position	1		2	
	δ_C	δ_H COSY	δ_C	δ_H COSY
1	105.8 (d)	6.62 d, 1.5	107.0 (d)	6.67 d, 1.9
1a	146.1 (s)	-	135.6 (s)	-
2	166.1 (s)	-	164.8 (s)	5.75 (OH)
3	99.3 (d)	6.39 d, 1.5	104.1 (d)	6.80 d, 1.9
4	164.4 (s)	12.0 s (OH)	164.7 (s)	
4a	108.0 (s)	-	115.5 (s)	-
5	198.2 (s)	-	193.7 (s)	-
5a	78.2 (s)	7.0 br (OH)	79.8 (s)	5.8 br (OH)
6	73.9 (d)	5.29 d, 6.3	63.1 (d)	4.50 d, 2.5 3.3 br (OH)
7	81.4 (d)	4.67 d, 6.3	76.2 (d)	3.58 d 3.3 br (OH)
8	68.6 (s)	5.6 br (OH)	72.8 (s)	3.3 br (OH)
9	33.6 (t)	1.96 d, 16.5 2.12 d, 16.5	31.6 (t)	1.60 d, 4.9 2.12 d, 4.9
9a	74.2 (s)	6.0 br (OH)	79.8 (s)	5.8 br (OH)
10	69.1 (d)	4.65 d,br 5.6 br (OH)	197.5 (s)	-
11	26.8 (q)	1.30 s	27.2 (q)	1.20 s
12	55.7 (q)	3.80 s	55.8 (q)	3.81 s
13	154.5 (s)	-	-	-

Abbreviations: s; singlet, d; doublet, t; triplet, br; broad, q; quartet.

protons (6.67 ppm, 6.80 ppm), one methoxyl (3.81 ppm), one methyl (1.20 ppm), one methylene group (1.60 ppm, 2.12 ppm). However only two protons were visible attributable to oxygen-bonded carbons (3.58 ppm, 4.50 ppm). Two hydroxyl proton signals appeared at 3.30 ppm and 5.80 ppm. In contrast to **1** no signal was observed at 12.0 ppm advocating that in **2** the methoxyl group (O–C-12) is bound to C-4 of the aromatic ring. The ^{13}C and DEPT spectra of **2** unveiled the presence of six aromatic carbons, two quinone carbonyles, one methylene, one methyl, one methoxyl and five oxygen-bonded carbons. Amongst the latter there were three quaternary carbons (C-5a, C-8, C-9a). Moreover, the signal appearing in **1** at 154.5 ppm was missing in the ^{13}C NMR spectrum of **2** in accord with the absence of a cyclic carbonate structure.

Conclusive evidence for the structure of **1** and **2** as shown in Fig. 1 was furnished by the 2D NMR spectra (COSY, HMBC, NOESY). Instructive C,H long-range and NOESY connectivities are shown in Fig. 2.

For the assignment of structures $^{2}J_{C,H}$ and $^{3}J_{C,H}$ couplings of the aromatic, methylene, methyl and methoxyl protons were of pivotal importance. Couplings of H-1 and H-3 with the aromatic carbons, C-5 and C-10 settled the constitution of this part of the molecules. The positions of C-5 and C-10 relative to the aromatic ring and the substituted cyclohexane was assignable due to the C–H long-range couplings of H-6 and C-5. COSY and HMBC correlations settled doubtlessly the positions of the other substitutions. The relative stereochemistry of **1** and **2** as shown in Fig. 3 was proposed on the basis of strong NOE between H-11 and H-7, missing NOE correlation between H-11 and H-6, and the values of $^{3}J_{H\text{-}6/H\text{-}7}<8$ Hz. Thus for C-11 an equatorial position can be suggested due to its steric requirements. As a consequence of the observed NOE, H-7 should occupy an axial position, as this was shown for most of the altersolanol-type structures, and H-6 should be equatorial due to $J_{H\text{-}6.H\text{-}7}=6.3$ Hz. The constitution of **1** was confirmed further by the observable NOE between H-1 and H-10. However, due to invisible NOESY correlations of HO-5a and HO-9a with neighboured protons the relative stereochemistry of **1** and **2** at these positions was not assignable.

Chrysoqueen (**1**) and chrysolandol (**2**) thus appear as new members of the naphthaquinone-type altersolanol family of antibiotics which are known as products of fungi such as *Alternaria solani*[3], *Dactylaria lutea*[2], *Phomopsis*

Fig. 1. Structures (relative stereochemistry) of **1** and **2**.

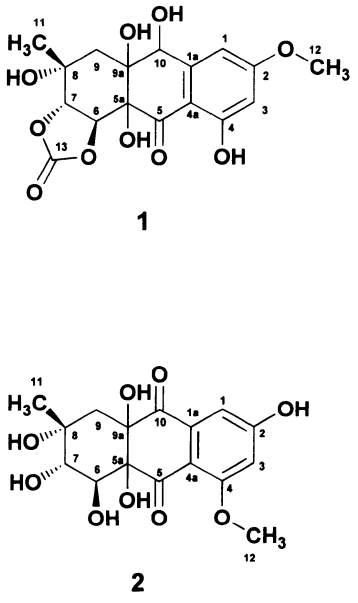
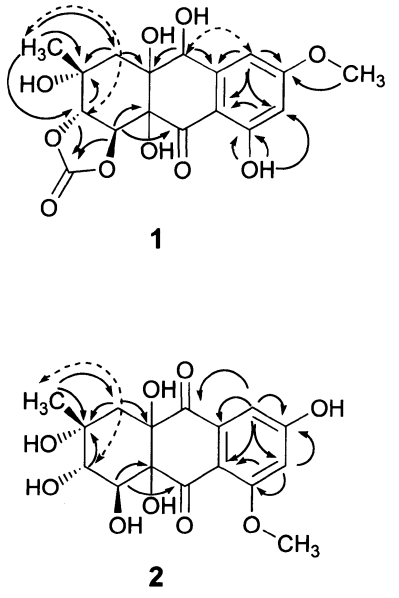


Fig. 2. Instructive C,H long-range (⟶) and NOE connectivities (⟷) in the HMBC and NOESY spectra of **1** and **2**.



juniperivora[1], *Fusarium solani*[5,6], *Nectria haematococca*[7] and *Torula herbarum*[9,10]. Antibiotic and antitumor activities were reported for fungal naphthaquinones[11,12].

The new compounds **1** and **2** showed activity against Gram-positive bacteria such as *Micrococcus luteus* IFM 2066 (MIC 33 μg/ml) and *Bacillus subtilis* PCI 219 (33 μg/ml) but were inactive against Gram negative bacterium, yeast and filamentous fungi such as *e.g. Escherichia coli* NIH JC-2, *Candida albicans* ATCC 90028 and *Paecilomyces variotii* IFM 40913 and *A. niger* IFM 5368. However in comparison with coproduced altersolanols A, B, and C the antimicrobial activity of **1** and **2** was lower.

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