Queenslandon, a New Antifungal Compound Produced by *Chrysosporium queenslandicum*: Production, Isolation and Structure Elucidation

YASUTAKA HOSHINO, VENETA BOJANOVA IVANOVA†, KATSUKIYO YAZAWA, AKIKAZU ANDO and YUZURU MIKAMI*

Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, 1-8-1, Inohana, Chuo-ku, Chiba 260-8673, Japan

SHERIF MOHAMED ZAKI, AL-ZAHRAA A. KARAM and YOUSSEF A. YOUSSEF

Department of Microbiology, Faculty of Science, Ain-Sham University, Abbasia, Cairo, Egypt

UDO GRÄFE

Hans-Knöll-Institute for Natural Products Research, Beutenbergstrasse 11, D-07745 Jena, Germany

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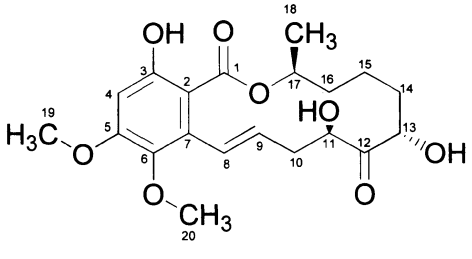
In the course of our screening program for biologically active compounds from pathogenic microorganisms, an antibacterial and antifungal naphthoquinone group antibiotic complex was isolated from the culture broth of a strain *Chrysosporium queenslandicum* IFM 51121. The compounds of the complex were identified as altersolanol A, B and C[1~3]. Besides the antibiotics, the strain *C. queenslandicum* IFM 51121 produces also a new antifungal compound related to zearalenone family of mycotoxin[4], designated queenslandon (**1**).

In this paper we report the production, isolation and structure elucidation of the mycotoxin queenslandon (Fig. 1), through the use of one and two dimensional NMR techniques and mass-spectrometry.

C. queenslandicum IFM 51121 strain was isolated from a soil sample collected in Egypt, and deposited in the collection of 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 flask was 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 standing cultivation at 36°C, the culture broth (7.5 liters) of *C. queenslandicum* IFM 51121 was centrifuged. The supernatant fluid was passed through a Diaion HP 20 column (5×15 cm) and the active fractions against *Aspergillus niger* IFM 5328 and *Paecilomyces variotii* IFM 40913 were eluted with MeOH : H_2O (1 : 1 and 1 : 0). The methanol fraction was concentrated *in vacuo*, after that suspended in water (350 ml) and extracted three times with ethyl acetate (200 ml). The combined ethyl acetate extract was filtered and evaporated to dryness *in vacuo*, yielding 150 mg crude product (queenslandon). The ethyl acetate solution of the crude product was chromatographed on a silica gel FL100D column equilibrated with chloroform. Queenslandon was eluted from the column, using a gradient of 20% to 90% methanol in chloroform. The queenslandon containing fraction showed a positive color reaction to molybdo(VI)phosphoric acid on TLC plates with $CHCl_3$: MeOH (95 : 5, v/v) as the mobile phrase. The active fraction was dissolved in a small amount of chloroform and further purification was achieved by preparative thin-layer chromatography on silica gel plate with $CHCl_3$: MeOH (95 : 5, v/v) as the mobile phase. The compound was eluted with chloroform. The complete separation and purification of queenslandon could be achieved by preparative HPLC on a (250×5 mm) megapak SILC 18T-10 column, using a gradient of 20% to 90% acetonitrile in water and monitoring at 220 nm, RT=13.04. The purified fractions were concentrated *in vacuo* until removal of acetonitrile and extracted with chloroform. The chloroform extract was concentrated *in vacuo*. Yields

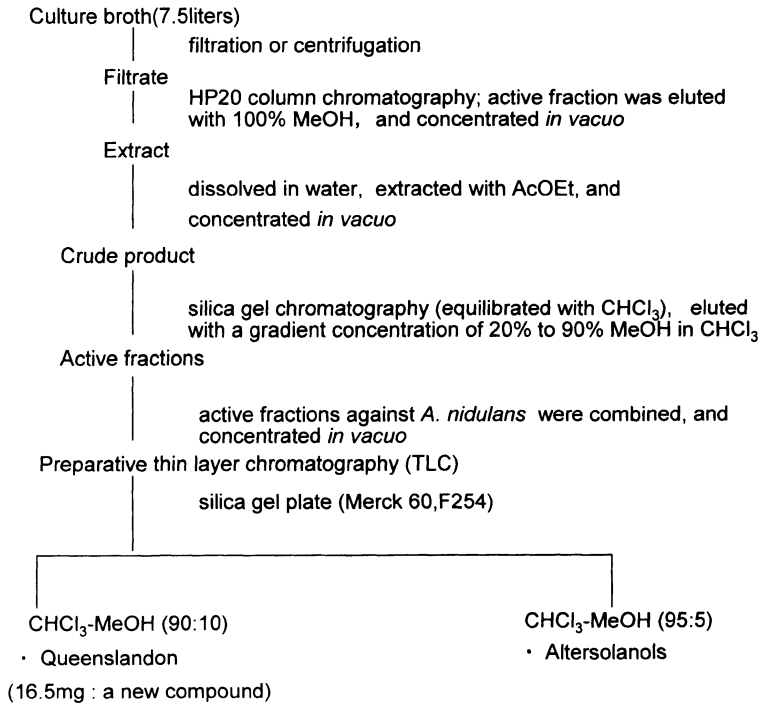
Fig. 1. Structure of queenslandon (relative stereochemistry).



† Present address: Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria

* Corresponding author: mikami@myco.pf.chiba-u.ac.jp

Fig. 2. Scheme for the isolation of pure queenslandon.



(20 mg) of queenslandon was crystallized from methanol. The scheme for the isolation of pure queenslandon is shown in Fig. 2.

Queenslandon was isolated as white microcrystals. It is optically active with $[\alpha]_D^{25}$ +24.4° (methanol concentration was 0.28 mg/ml, 1.0 cm cuvette). The melting point was 157~159°C. The substance is soluble in chloroform, partially in ethyl acetate, but insoluble in lower alcohols and water. On thin-layer plates queenslandon showed positive reaction with molybdo(VI)phosphoric acid yielding a blueish color. It gave a single spot on TLC (silica gel; $CHCl_3$: MeOH 95 : 5 and 80 : 20 (v/v), suggesting Rf values of 0.38 and 0.45 in each solvent system).

The structure of queenslandon (**1**) as a new representative of the zearalenone family[4~8] of mycotoxin lactones was settled on the basis of optical spectroscopy (IR), electrospray (ESI-MS) and high-resolution electron impact mass spectrometry (HREI-MS), one and two dimensional NMR spectroscopy (^1H, ^{13}C, DEPT, COSY, HMQC, HMBC, NOESY and TOCSY). The assignment of proton and ^{13}C signals is shown in Table 1.

In the IR spectrum of **1** (film) absorbances at 1705 cm^{-1} and 1644 cm^{-1} attested to the presence of carbonyls and an aromatic system, respectively. Moreover, diagnostically useful λ_{max} values were observed at 754, 808, 830, 912, 970, 1018, 1063, 1119, 1169, 1203, 1225, 1247, 1314, 1358, 1385, 1429, 1473, 1595, 1937 and 3370 (OH) cm^{-1}.

The positive ion electrospray mass spectrum displayed *m/z* 417.3 ($[M+Na]^+$) and the negative ion mode *m/z* 393.3 ($[M-H]^-$). The molecular weight (394 Da) and the chemical formula $C_{20}H_{26}O_8$ were readily determined by HREI-MS due to *m/z* 394.1625 (M^+; calcd. 394.1628). The formula suggested the presence of eight double bonds or rings in the molecule.

In the ^{13}C NMR spectrum eight doubly bonded carbons were visible. Three of them were doublets according to the DEPT spectrum indicating the occurrence of a pentasubstituted benzene ring and an additional double bond. Moreover, a keto group (C-12; 209.7 ppm) and an ester carbonyl (C-1; 171.1 ppm) were visible. Due to 161.4 ppm, 158.9 ppm and 140.1 ppm three of the aromatic carbons (C-3, C-5, C-6) should be bonded to oxygen. Also the chemical shift of three aliphatic carbons (79.8 ppm (C-11), 73.0 ppm (C-13), 74.2 ppm (C-17)) unveiled the presence of further oxygens in the molecule. The aromatic proton at 6.40 ppm (H-4) appeared as singlet. Two other double bond protons (H-8: 6.61 ppm; H-9: 6.02 ppm) were coupled with $^3J_{H,H}$=14.8 Hz suggesting *E*-configuration.

Table 1. Assignment of ^{1}H and ^{13}C NMR signals of **1** (500 MHz, in $CDCl_3$, chemical shifts in ppm, TMS as internal standard, coupling constants in Hz, s: singlet, d: doublet, t: triplet, m: multiplet, br: broad).

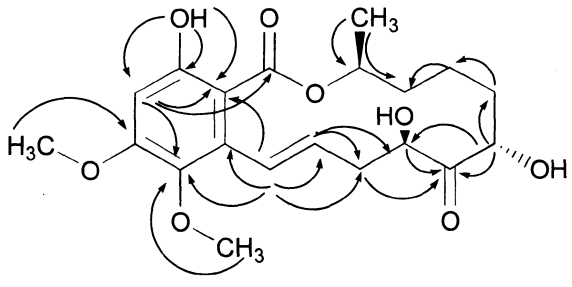
position	δ_C	δ_H
1	171.1	-
2	103.6	-
3	161.4	11.84 (OH)
4	99.4	6.40 s
5	158.9	-
6	140.1	-
7	132.2	-
8	126.9	6.61 d; 14.8
9	130.4	6.02 ddd; 14.8; 10.0; 3.1
10	37.9	2.7; 3.1; 7.6; 3.2; 2.1 ddd; 10.0; 5.8; 7.6
11	79.8	4.1 dd; 5.8; 3.2; 3.6 br (OH)
12	209.7	-
13	73.0	5.18 ddd; 3.6; 6.0; 2.1; 3.60 br (OH)
14	20.9	1.46 m; 1.75 m
15	34.9	1.70 m
16	39.5	2.4 m; 2.7 m
17	74.2	3.95 m
18	20.0	1.38 d; 6.8
19	55.9	3.80 s
20	60.2	3.58 s

The down-field shift of the phenolic proton signal at 11.84 ppm indicated its β-position relative to the ester carbonyl (C-1). Further, a bulk of methylene protons (H-14, H-15, H-16) and signals of H-10 and H-17 protons were visible. The doublet methyl signal at 1.38 ppm was readily explained by confirmed the neighbourhood of C-17. Two singlet methoxyl proton signals at 3.80 ppm (H-19) and 3.58 ppm (H-20) were further diagnostic features proving the structure of **1** as shown in Fig. 1.

For the structural assignment of queenslandon **1** the $^{1}H,^{1}H$-COSY and C,H long-range heteronuclear coupled NMR spectra (HMBC) were of pivotal importance. The instructive C, H-connectivities in the HMBC spectrum of **1** are shown in Fig. 3. Visible $^{2}J_{C,H}$ and $^{3}J_{C,H}$ couplings of H-4 and of the methoxyl protons with the neighboured carbons settled the substitution pattern at the aromatic ring. A weak $^{4}J_{C,H}$ coupling of H-4 with C-1 was in accord with this contention.

The sequence of protons and carbons in the lactone ring was doubtlessly assignable. In the C, H-coupled long range spectrum (HMBC) couplings of H-8 and H-9 with C-7, at the one hand, and of H-9 with C-10 and C-11, respectively, and of H-11 and H-13 with C-12, on the other hand, proved the comparably unusual position of the carbonyl group (C-12) between C-11 and C-13. This suggestion was confirmed

Fig. 3. Instructive C, H-correlations in the HMBC spectrum of queenslandon.



by the COSY NMR spectrum showing $^{3}J_{H,H}$ couplings of H-9/H-10 and H-10/H-11 but not between H-11 and H-13. The relative configuration at C-13 and C-17 was settled on the basis of the NOESY spectrum showing NOE's between H-13 and H-18 but not between H-11 and H-13.

The literature value comparison between the structures of queenslandon and the mycotoxin L-783279[9,10] showed that the both substances are very similar. The difference is in the presence of one methoxy group more in position C-6 and the different position of a keto group (C-12) by queenslandon.

The new substance as natural product was found in the culture broth of the strain *C. queenslandicum* IFM 51121, and showed activity against fungi but not bacteria such as *Micrococcus luteus* IFM2066 and *Bacillus subtilis* PCI 219. The antifungal activity was characteristic and the fungal growth in the inhibition zone around a paper dics (8 mm in diameter) was partial and not completely stopped. The maximum inhibition diameter by the 8 mm paper disc containing 50 μg of the compound against *Aspergillus nidulans* IFM 5369 was 52.1 mm. Moderate antifungal activities against *Alternaria alternata* IFM 41348, *Paecilomyces variotii* IFM 40913, *Penicillium chrysogenum* IFM 40614, *Aspergillus flavus* IFM 41934, *Aspergillus fumigatus* IFM 41088, *Aspergillus terreus* IFM 40851, and *Aspergillus niger* IFM 5368 were also confirmed. In this assay system, the inhibition diameters of the reference drug amphotercin B against *Aspergillus niger* IFM 5368 and *Aspergillus nidulans* IFM 5369 were 13 and 32 mm, respectively.

Fungal metabolites such as zearalenones containing a lactone function in their molecules are of interest because of their strong biological activity such as estrogenic activity or structural similarity to carcinogenic fungal lactones[11]. Now detail toxicity studies on queenslandon is in progress in our laboratory.

Acknowledgments

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