Griseusin D, a New Pyranonaphthoquinone Derivative from a Alkaphilic *Nocardiosis* sp.

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Abstract A new pyranonaphthoquinone antibiotic, griseusin D (**1**) was isolated from the cultural fluid of the alkaphilic *Nocardiosis* sp. The structure was determined as 5'-one-4-hydroxy-12-methoxygriseusin by spectroscopic methods, comparison with reported data and single-crystal X-ray analysis. **1** displayed strong cytotoxicity against human leukemia cells (HL60) and modest cytotoxicity against human lung adenocarcinoma cell lines (AGZY) with IC₅₀ values of 0.23 and 19.6 µg/ml, respectively. It also exhibited weak antifungal activity against *Alternaria alternata* with MIC of 140 µg/ml.

Keywords pyranonaphthoquinone, griseusin D, alkaphilic *Nocardiosis* sp.

Pyranonaphthoquinone antibiotics isolated from various strains of bacteria and fungi have been shown to exhibit activity against a variety of Gram-positive bacteria, pathogenic fungi and yeasts, as well as exhibiting antiviral activity [1]. As unique members within the family of pyranonaphthoquinone antibiotics, griseusins A, B, C and their derivatives have been isolated from the cultures of *Streptomyces griseus* K-63 [2–4], *S. griseus* MJ361-48F3 [5], Actinomycete strain MJ932-SF3 [6] and *Penicillium* sp. [7].

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In the course of our searching for novel anticancer agent, we have isolated a new antibiotic, griseusin D (**1**, Fig. 1), from the culture of alkaphilic *Nocardiosis* sp. YIM 80133, which was isolated from soil sample collected from Qinghai Province, China. In this paper, we report the fermentation, isolation, structure elucidation and cytotoxicity of **1**.

Fermentation studies of the culture YIM 80133 were carried out in shake flasks. Stock cultures were maintained as frozen whole broths as –80°C in a final concentration of 10% glycerol. The germination medium contained glucose 4.0 g, yeast extract 4.0 g, malt extract 5.0 g, multiple vitamins solution 5.0 ml, and trace element solution 1.0 ml per liter. The pH was 7.2 with no adjustment. A 500-ml Erlenmeyer flask containing 100 ml of this medium was inoculated with 3.0 ml of the stock culture. The flasks were incubated at 28°C on a rotary shaker at 280 rpm for 72 hours.

This germination (30 ml) was used to inoculate the fermentation medium. The fermentation was carried out in a 500-ml Erlenmeyer flask containing 100 ml of fermentation medium. The fermentation media used contained soybean flour 20 g, and mannitol 20 g per liter.

Fig. 1 Structure of griseusin D (**1**).

Fig. 2 LC-MS analysis of filtrate of fermentation broth of YIM 80133.

Peak at $R_t=15.06$ minutes in LC and its molecular ion m/z 431.1025 $[M-H]^-$ in TOF MS verified that the griseusin D was present in the culture before exposure to MeOH. The filtrate was concentrated by 10 times in volume at 40°C *in vacuo* and then subjected for LC-MS analysis. LC Condition: 1. Column: Waters XTerra MS C18, $3.5\ \mu\text{m}$, $3.0\times 150\ \text{mm}$. 2. Mobile Phase (Gradient): Acetonitrile : Water = 0 : 100 to 100 : 0.3. Time: 30 minutes. 4. Flow rate: 0.7 ml/minute. 5. Temperature: 30°C . 6. Wavelength: 254 nm.

The pH was adjusted to 9.5 with 1 M NaOH. The flasks were incubated at 28°C on a rotary shaker at 280 rpm for 120 hours.

The completed fermentation broth (30 liters) was separated into filtrate and mycelium by centrifugation. LC-MS analysis of filtrate (pH 6.58) confirmed that the griseusin D was present in the culture (Figs. 2 and 3). The culture filtrate was absorbed onto the polymeric resin Amberlite XAD-16 (8.0 liters, Rohm & Hass, France). The salt and high molecular materials were washed out with water followed by 50% aq MeOH (8.0 liters each). Then, other absorbed organic material was eluted with 8.0 liters 70% aq MeOH, and finally material was eluted with 100% MeOH to yield 13 g of dried material after removing solvent *in vacuo*. The organic material of the 100% MeOH fraction was purified by column chromatography on: (1) silica gel (CHCl_3 -MeOH gradient), (2) Sephadex LH-20

(MeOH), and by (3) preparative HPLC (Alltech Econosphere C18 $10\ \mu\text{m}$, $22\times 250\ \text{mm}$ i.d., MeOH- H_2O gradient, 10 ml/minute) to afford **1** (80.0 mg).

The structure of **1** was mainly elucidated by extensive 1D and 2D NMR data analysis. In the ^1H -NMR spectrum, a total of 17 carbon-attached protons were counted. One methyl, one methoxy, two methylene, and four oxygen-bearing methine signals were observed in aliphatic region, and three resonances were observed in the olefinic/aromatic region. In the ^{13}C -NMR spectrum, 21 carbon signals were detected, in which one cyclohexanone carbonyl group (C-5', δ 203.7), two conjugated ketone functionalities (C-5, δ 182.0; C-10, δ 187.8), and one ester carbonyl group (C-12, δ 171.0) were identified. The molecular ion m/z 455.0999, $[M+\text{Na}]^+$ was observed on an ESI-MS instrument, and therefore the molecular formula of **1** was calculated as $\text{C}_{21}\text{H}_{20}\text{O}_{10}$. Thus, three hydroxyl groups were proposed to

Fig. 3 Magnified spectrum of -TOF MS for 15.196 to 15.213 minutes of filtrate of fermentation broth of YIM 80133.

be presented in the molecule. The ^1H -NMR (H-6, 7.57, d, $J=7.4$ Hz; H-7, 7.78, t, $J=8.1$, 7.4 Hz; H-8, 7.38, d, $J=8.1$ Hz) and ^1H - ^1H COSY correlations from H-6 through H-8 revealed the presence of one trisubstituted benzene in the molecule. Based on HMBC correlations from H-8 (δ 7.38) to C-9a (δ 114.9) and C-6 (δ 118.5), H-7 (δ 7.78) to C-5a (δ 131.5) and C-9 (δ 160.5), H-6 (δ 7.57) to C-10 (δ 187.8), C-9a (δ 114.9), C-8 (δ 124.6), C-5 (δ 182.0) and C-4a (δ 136.8), and 9-OH (δ 11.23) to C-9a (δ 114.9), C-9 (δ 160.5) and C-8 (δ 124.6), the structure of fragment A was proposed (Fig. 4). Based on ^1H - ^1H COSY correlations from H-7' (δ 1.25) through H-3' (δ 4.28) and H-4' (δ 2.59, 2.42), and from H-11 (δ 2.81, 2.69) through H-3 (δ 4.31), H-4 (δ 4.45) and 4-OH (δ 4.98); as well as HMBC relations from H-7' (δ 1.25) to C-4' (δ 47.7) and C-5' (δ 203.7), H-4' (δ 2.59, 2.42) to C-7' (δ 21.1), C-6' (δ 76.3) and C-5' (δ 203.7), H-6' (δ 5.13) to C-1' (δ 100.2), H-13 (δ 3.62) to C-12 (δ 171.0), H-11 (δ 3.62) to C-12 (δ 171.0) and C-4 (δ 58.5), H-3 (δ 4.31) to C-12 (δ 171.0) and H-4 (δ 4.45) to C-10a (δ 145.3), the structure of fragment B was proposed (Fig. 4). Comparisons of the ^1H -, ^{13}C -NMR data and HMBC spectra (Fig. 4) of **1** and 4'-deacetyl-($-$)-griseusin B [6] showed that the compound **1** is very similar to the 4'-deacetyl-($-$)-griseusin B except for the signals of

Fig. 4 Partial structures, ^1H - ^1H COSY and key HMBC correlations of **1**.

Table 1 NMR spectral data for griseusin (**1**) in DMSO- d_6^a

C/H no.	^1H (δ)	^{13}C (δ)	^1H - ^1H COSY	HMBC
1		100.2 s		
3	4.31, 1H, m	67.9 d	H-4, H-11	C-12
4	4.45, 1H, d, $J=9.5$	58.5 d	H-3, 4-OH	C-10a
4a		136.8 s		
5		182.0 s		
5a		131.5 s		
6	7.57, 1H, d, $J=7.4$	118.5 d	H-7	C-4a, C-5, C-8, C-9a, C-10
7	7.78, 1H, t, $J=8.1, 7.4$	137.0 d	H-6, H-8	C-5a, C-9
8	7.38, 1H, d, $J=8.1$	124.6 d	H-7	C-6, C-9a
9		160.5 s		
9a		114.9 s		
10		187.8 s		
10a		145.3 s		
11a	2.81, 1H, d, $J=3.7$	34.7 t	H-3	C-4, C-12
11b	2.69, 1H, d, $J=8.8$			
12		171.0 s		
13	3.62, 3H, s	51.5 q		C-12
3'	4.28, 1H, m	67.5 d	H-4', H-7'	
4'a	2.59, 1H, d, $J=13.2$	47.7 t	H-3'	C-5', C-6', C-7'
4'b	2.42, 1H, d, $J=14.0$			
5'		203.7 s		
6'	5.13, 1H, s	76.3 d		C-1'
7'	1.25, 3H, d, $J=5.9$	21.1 q	H-3'	C-4', C-5'
4-OH	4.98, 1H, d, $J=9.5$		H-4	
9-OH	11.83, 1H, s			C-8, C-9, C-9a
6'-OH ^b	3.35, 1H, s			

^a The spectra were recorded on a Bruker 500 NMR instrument at 500 MHz for ^1H and 125 MHz for ^{13}C , δ in ppm, J in Hz. The solvent signals (2.50 ppm for ^1H -NMR, and 39.5 ppm for ^{13}C -NMR) were used as reference. ^b Overlap under H_2O signal.

cyclohexanone carbonyl at C-5', hydroxyl at C-4 and methoxy at C-13. Thus, the structure of **1** was elucidated as 5'-one-4-hydroxy-12-methoxygriseusin B, which we named griseusin D (Fig. 1). The unambiguous assignment of the ^1H and ^{13}C chemical shifts was achieved based on 2D NMR data analysis including ^1H - ^1H COSY, HSQC, and HMBC as detailed in Table 1.

The stereochemistry of **1** was established by analyses of NOESY. The relative stereochemistry on C-3, C-4, C-3', C-6' was assigned on the basis of the NOE correlations between H-3 and H-4, H-3' and H-4'a or H-4'b, H₃-7' and H-4'a or H-4'b, 4-OH and 6'-OH as shown in Fig. 5. Furthermore, the constitutional formula as well as the relative configuration of **1** derived from the interpretation of the NMR data was fully confirmed by single-crystal X-ray determination. A perspective view of the solid state conformation was shown in Fig. 6.

Crystals of **1** were obtained from a hot MeOH/H₂O

Fig. 5 The relative configuration of **1** and NOE correlation observed in NOESY spectrum (represented by double arrows).

solution. A yellow needle crystal of 0.22×0.20×0.16 mm was mounted on a glass fiber. All measurements were made on a Bruker SMART APEX CCD X-ray diffractometer

Fig. 6 Crystal structure of **1**.

with graphite monochromated Mo-K α radiation. Crystal data: Empirical formula: C₂₁H₂₀O₁₀; Molecular weight: 432.37; Crystal system: monoclinic; Space group: P2(1); Lattice parameters: $a=15.961(3)$ Å, $b=7.8084(15)$ Å, $c=17.606(3)$ Å; Volume: 2087.3(7) Å³; Z value: 4; D_{calc}: 1.376 g/cm³; T: 293 K. The structure was solved by a direct method (SHELXS-97). Final R and wR were 0.0616 and 0.1531 for 5911 observed reflections, respectively.

1: Orange needle crystal; HRESI-MS m/z 455.0999 [M+Na, C₂₁H₂₀O₁₀Na]⁺, 431.1025 [M-H, C₂₁H₁₉O₁₀]⁻; $[\alpha]_D^{26}$ -68 (c 0.005, MeOH); UV $\lambda_{\max}^{\text{MeOH}}$ nm (log ϵ) 213 (3.51), 256 (3.74); ¹H- and ¹³C-NMR data, see Table 1.

1 displayed strong cytotoxicity against human leukemia cells (HL60) and modest cytotoxicity against human lung denocarcinoma cell lines (AGZY) with IC₅₀ values of 0.23 and 19.6 µg/ml, respectively. It also exhibited weak antifungal activity against *Alternaria alternate* with MIC of 140 µg/ml.

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