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NOTE

A new cytotoxic and anti-fungal C-glycosylated benz[α]anthraquinone from the broth of endophytic *Streptomyces blastomycetica* strain F4-20

He Yan, Yang Li, Xing Y Zhang, Wan Y Zhou and Tao J Feng

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Endophytes reside in the tissues of living plants without exerting any pathogenic effects.[1] Endophytes produce a great number of secondary metabolites with diverse chemical structures and various biological activities, which have been implicated in the protection of their hosts against pathogens and herbivores.[2] Endophytic microorganisms are an excellent source of structurally diverse molecules with potential therapeutic value.

In this study, a new C-glycosylated benz[α]anthraquinone, dehydroxyaquayamycin B (**1**), along with two known alkaloids, teleocidin B2 (**2**) and *N*-methyl-L-valyl-L-tryptophanol (**3**), was isolated from the endophytic *Streptomyces blastomycetica* strain F4-20 (Figure 1). The new compound was tested for cytotoxic and anti-fungal activities. The strain F4-20 was isolated from the root of *Tripterygium wilfordii* Hook. f., a medicinal plant in China, by spreading on actinomyces isolation agar from Difco (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), consisting of 0.05% dipotassium phosphate, 0.0001% ferrous sulfate, 0.01% magnesium sulfate, 0.05% sodium chloride, 0.1% potassium nitrate, 0.2% soluble starch and 1.5% agar, pH 7.2–7.4, and incubated at 28 °C for 7–14 days. The strain was identified as a member of the genus *Streptomyces* because its 16S rRNA sequence exhibited 99% similarity to *Streptomyces spectabilis* NRRL B-5480, and has been deposited in the Research and Development Center of Biorational Pesticide, Northwest A&F University, with the accession no. F4-20.

The strain was cultivated on the optimized solid medium containing glucose (Bei Jing Ao Bo Xing, Beijing, China) 20 g, potato 200 g, beef extract 10 g, KH_2PO_4 1 g, ammonium sulfate 1 g, $NaNO_3$ 1 g, NH_4Cl 1 g and agar 17 g in 1.0 L tap water, pH 8.0–8.5. The spore suspension was then filtered through six layers of sterile filter cheesecloth and adjusted to 10^7–10^8 CFU ml^{-1}. A 2.0 ml of the spore suspension was inoculated into a 250-ml flask containing 100 ml of seed medium consisting of glucose (Bei Jing Ao Bo Xing) 4 g, malt extract powder (Cormwin, Beijing, China) 10 g and yeast autolysate (Bei Jing Ao Bo Xing) 4 g in 1.0 L tap water, pH 7.3, and incubated at 28 °C for 24 h, with shaking at 140 r.p.m. Then, each 8.0 ml of the cultured seed liquid were transferred into 340 250-ml Erlenmeyer flasks containing 150 ml of the sterile fermentation medium consisting of glucose (Bei Jing Ao Bo Xing) 0.4%, malt extract powder (Cormwin, Beijing, China) 1% and yeast autolysate (Bei Jing Ao Bo Xing) 0.4%, pH 7.3. Fermentation was carried out at 28 °C for 7 days on a rotary shaker at 140 r.p.m.

The final 50 L of broth was filtered and evaporated under reduced pressure to 1 L at 55 °C and the resulting concentrate was extracted three times using an equal volume of EtOAc. The EtOAc-soluble fraction (9.5 g) was applied to silica gel column (200–300 mesh; Qingdao Marine Chemical, Qingdao, China) eluting with a $CHCl_3$-MeOH gradient (10:0, 20:1, 9:1, 8:2, 7:3, 1:1 and 0:1) to give five fractions A–E. The separation of fraction C (2.4 g) over silica gel column (200–300 mesh; Qingdao Marine Chemical) was eluted with petroleum ether–acetone (30:1–4:1) to yield fractions C-1–C-7. Fraction C-3 (0.21 g) was subjected to a reversed-phase column (RP-18) eluting with MeOH–water (20–90%) to afford four subfractions (C-3-1–C-3-4). C-3-2 (0.08 g) was subjected to semipreparative reversed-phase HPLC (Shimadzu LC20A apparatus equipped with a UV detector and a Hypersil BDS C_{18} (Thermo, Shanghai, China; 250×10 mm^2)) to give **1** (29.7 mg) and **2** (2.4 mg). C-3-3 (0.017 g) was subjected to semipreparative reversed-phase HPLC to give **3** (1.4 mg).

Compound **1** was isolated as an optically active amorphous red solid. Its molecular formula $C_{37}H_{42}O_{11}$ was determined by the ESI-HRMS (API QSTAR Pulsar mass spectrometer; VG, Manchester, UK), owing to the presence of a pseudomolecular ion peak at *m/z* 685.2623 $[M+Na]^+$ (calcd. for $C_{37}H_{42}O_{11}Na$, 685.2625). The IR spectrum (*Tensor 27* FT-IR spectrometer with KBr pellets) showed absorption bands at 3387, 1686, 1590, 1281 and 1268 cm^{-1}, indicative of the existence of hydroxyl and carbonyl groups. Analysis of the ^{13}C and DEPT NMR spectra (*DRX-500*, ^1H: 500 MHz; ^{13}C: 125 MHz) revealed the presence of two ketone carbonyl groups (δ_C 188.25, C-7 and δ_C 189.93, C-12), 10 sp^2 quaternary, 6 sp^2 methine, 10 sp^3 methine, 5 sp^3 methylene and 4 sp^3 methyl carbons. The ^1H NMR spectrum of **1** also showed 19 well-resolved resonances that comprised of 6 sp^2-hybridized methines, 10 sp^3-hybridized methines, 5 sp^3 methylene and 4 sp^3-hybridized methyls, consistent with an

Research & Development Center of Biorational Pesticide, Northwest A&F University, Yangling, China
Correspondence: Professor JT Feng, Research and Development Center of Biorational Pesticide, Northwest A&F University, Yangling 712100, China.
E-mail: fengjt67@hotmail.com
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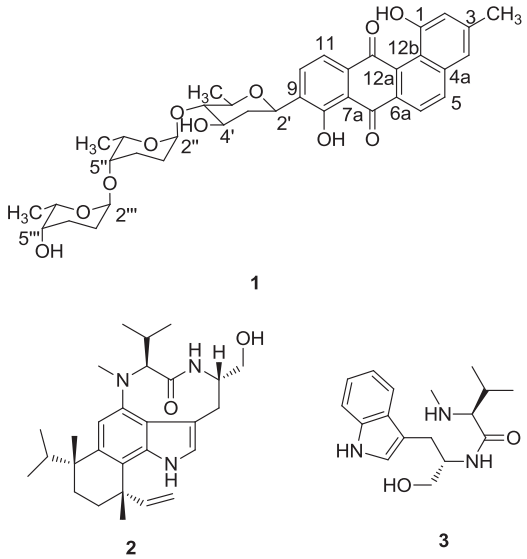

Figure 1 Chemical structures of dehydroxyaquayamycin B (**1**), teleocidin B2 and *N*-methyl-L-valyl-L-tryptophanol.

angucycline core and three glycosidic residues. Two broad singlets at δ_H 12.70 and 11.45, representing *peri*-hydroxy groups, and two 1,2,3,4-tetrasubstituted aromatic moieties were revealed by two AB systems (δ_H 7.92 and δ_H 7.97, δ_H 8.18 and δ_H 8.36). Two additional broad aromatic signals, each ^{1}H, at δ 7.19 and 7.30, showed another highly substituted aromatic ring with two *m*-coupled aromatic protons. Furthermore, a singlet of an aromatic-bound methyl group was observed at δ_H 2.54. The ^{1}H and ^{13}C NMR also revealed the presence of three saccharide moieties (three anomeric ^{1}H singlets, δ_H 5.10–4.89) (Table 1). The HMBC spectrum displayed correlations from the sp^3 methine proton at δ_H 4.96 (H-2′) to two carbons at δ_C 137.9 (C-9) and 133.3 (C-10), suggesting a C-glycosidic linkage. Further examination of these NMR data exhibited comparability of dehydroxyaquayamycin,[3] which was originally obtained upon successive hydrogenation and acidification of aquayamycin. The only difference between compound **1** and dehydroxyaquayamycin was the longer saccharide moiety at C-9 position.

The saccharide moieties were deduced from detailed analyses of the one- and two-dimensional NMR data (^{1}H-^{1}H COSY, HMQC, HMBC and NOESY spectra) of **1**. The anomeric proton at δ 4.96 (d, J=11.2 Hz, H-2′) showed large coupling constant and thus represented β-D-glycoside moieties. The remaining two anomeric protons at δ_H 5.10 (brs, H-2″) and δ_H 4.89 (brs, H-2‴) were α-glycosidically linked L-sugars. The COSY spectrum revealed the spin systems extending from H-2′ to H-7′ and HMBC spectrum displayed correlations from the sp^3 methine proton at δ_H 4.87 (H-2″) to two carbons at δ_C 137.9 (C-9) and 133.3 (C-10). The latter suggested that this β-D-glycoside moiety was linked at the C-9 position. The dd peak at δ_H 3.25 (H-5′) gave coupling constants of 8.87 and 8.87 Hz, indicating the axial orientation. Hence, the methyl at C-7′ and two hydroxyl protons at C-4′ and C-5′ are in equatorial positions. NOESY spectrum, showing the cross-peak correlations from H_{ax}-2′ to H_{eq}-4′ and H_{ax}-6′, also confirmed the above assumption. Thus, the first hexose moiety that linked to C-9 by a C-glycosidic linkage was identified as a β-D-olivose unit. The COSY spectrum also revealed the spin systems extending from H-2″ to H-7″. Wide single peaks at 3.60 (H-5″) indicated the equatorial orientation of this proton. In consideration of NOESY cross-peak correlations from H_{ax}-3″ to H_{eq}-2″ and H_{ax}-6″, this hexose moiety could be deduced to L-rhodinose. The last sugar showed the same signal patterns and connectivity as L-rhodinose. HMBC correlations were used to establish the structure of the side chains and their points of attachment. The anomeric proton (δ_H 4.96) of the olivose moiety showed an HMBC correlation to C-9 (δ_C 137.9) and 133.3 (C-10) of the aglycone, whereas an HMBC correlation between the anomeric proton of the second L-rhodinose moiety (δ_H 5.10) and the carbon at δ_C 76.21 (C-5′), indicating that the second as rhodinose unit was attached to C-5′ of the olivose. The anomeric protons of the third L-rhodinose moiety (δ_H 4.89) showed HMBC correlations to C-5″ (δ_C 74.56) of the second L-rhodinose unit, indicating that the third as a rhodinose unit was attached to C-5″ of the second L-rhodinose (Figure 2). Thus, the

Table 1 ^{1}H and ^{13}C NMR spectral data (in acetone-d_6) of dehydroxyaquayamycin B

Position	*^{1}H NMR (p.p.m., J in Hz)*	*^{13}C NMR (p.p.m.)*
1		155.43 (s)
2	7.19 (s)	120.12 (d)
3		142.05 (s)
4	7.30 (s)	121.20 (d)
4a		134.86 (s)
5	8.18 (d, J=8.5 Hz, ^{1}H)	137.57 (d)
6	8.36 (d, J=8.6 Hz, ^{1}H)	121.85 (d)
6a		139.19 (s)
7		188.25 (s)
7a		114.01 (s)
8		157.94 (s)
9		138.54 (s)
10	7.97 (d, J=7.8 Hz, 2H)	133.58 (d)
11	7.92 (d, J=7.8 Hz, 2H)	121.40 (d)
11a		133.42 (s)
12		189.93 (s)
12a		132.52 (s)
12b		120.12 (s)
13	2.54 (overlap)	21.25 (q)
2′	4.96 (d, J=11.2 Hz, ^{1}H)	71.22 (d)
3′	1.54 (overlap)	37.59 (t)
	2.56 (overlap)	
4′	3.78 (^{1}H, m)	82.29 (d)
5′	3.25 (1H, dd, J=8.87, 8.87 Hz)	76.21 (d)
6′	3.56 (^{1}H, m)	76.21 (d)
7′	1.50 (^{3}H, d, J=5.8 Hz)	18.46 (q)
2″	5.10 (^{1}H, brs)	97.61 (d)
3″	1.62 (overlap)	25.45 (t)
	2.13 (overlap)	
4″	1.83 (overlap)	24.27 (d)
	2.05 (overlap)	
5″	3.60 (^{1}H, brs)	74.56 (d)
6″	4.19 (^{1}H, m)	67.97 (d)
7″	1.27 (^{3}H, d, J=6.4 Hz)	17.04 (q)
2‴	4.89 (^{1}H, d, J=1.5)	99.55 (d)
3‴	1.75 (overlap)	23.57 (t)
	2.00 (overlap)	
4‴	1.82 (overlap)	25.96 (t)
	2.12 (overlap)	
5‴	3.65 (^{1}H, brs)	67.46 (5)
6‴	4.08 (^{1}H, m)	66.84 (d)
7‴	1.21 (d, J=6.5 Hz, ^{3}H)	17.04 (q)

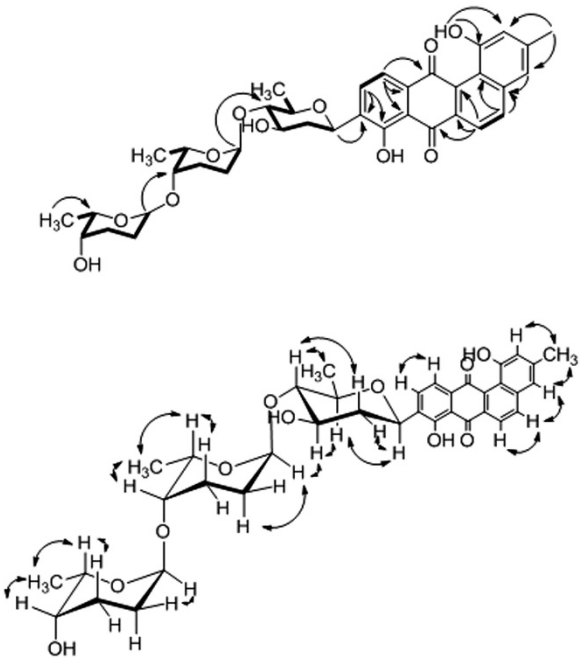

Figure 2 HMBC connectivities (→), $^{1}H,^{1}H$ COSY correlations (bold lines) and diagnostic NOESY couplings (←→) of dehydroxyaquayamycin B (**1**).

Table 2 Fungicidal activities of 1 with 50 μg ml^{-1}

Compound	*Fungals*	*Inhibition rate (%)*
1	*Valsa mali*	41.45
	Colletotrichum orbiculare	58.33
	Fusarium graminearum	51.00
	Rhizoctonia cerealis	1.02
	Botrytis cinerea	2.10
	Sclerotinia sclerotiorum	1.87
	Penicillium italicum	2.32

gross structure of **1** was assigned as shown in Figure 1, and this new compound was named as dehydroxyaquayamycin B.

Cytotoxicity of compound **1** against cancer cell lines BGC-823 and HeLa were tested using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Kit (Promega, Madison, WI). In these tests, dehydroxyaquayamycin B showed strong inhibitory activity on the proliferation of BGC-823 and HeLa cells with the half maximal inhibitory concentration (IC_{50}) value of 0.71 and 1.34 μg ml^{-1}, respectively.

Fungicidal activities of the compound **1** against *Valsa mali*, *Colletotrichum orbiculare*, *Fusarium graminearum*, *Rhizoctonia cerealis*, *Botrytis cinerea*, *Sclerotinia sclerotiorum* and *Penicillium italicum* were evaluated *in vitro* using the mycelium growth rate method[4] with 50 μg ml^{-1} (Table 2). The results are given in Table 2. Results indicated dehydroxyaquayamycin B showed appreciable anti-fungal activity against *V. mali*, *C. orbiculare* and *F. graminearum* with the inhibition rate of 41.5%, 58.3% and 51.0%, respectively.

Dehydroxyaquayamycin B is a benz[α]anthracene glycoside with a C-glycosidic substituent on C-9 position. Benz[α]anthraquinones have been isolated from various actinomycetes, especially in the genus *Streptomyces*. The first benz[α]anthraquinones, tetrangomycin and tetrangulol, were isolated from *Streptomyces rimosus* in 1966. These type of compounds have gathered attention because of their structural diversity and significant biological activity. Biological activities of benz[α]anthraquinones such as anti-*Helicobacter pylori*,[5] anti-fungal,[6] anti-Gram-positive bacterial,[7,8] anticancer,[9] anti-bacterial[10] activities have been reported. For example, urdamycins, isolated from *Streptomyces fradiae* strain Tu 2717, displayed biological activities including inhibition of platelet aggregation, anti-microbial activity for Gram-positive bacteria and anticancer against stem cells of murine L1210 leukemia.[9]

Naturally occurring C-glucosylated benz[α]anthraquinones at C-9 such as YM-181741 from *Streptomyces* sp. showed anti-*Helicobacter pylori* activity with an minimum inhibitory concentration value of 0.2 mg ml^{-1}, but was inactive against Gram-positive and -negative bacteria.[5] Urdamycinones E, G and dehydroxyaquayamycin exhibited antimalarial and antitubercular activities.[3]

Dehydroxyaquayamycin B exhibited considerably cytotoxic activities on the proliferation of BGC-823 and HeLa cells with the IC_{50} value of 0.71 and 1.34 μg ml^{-1}, respectively. It should be noted that naturally occurring C-glucosylated benz[α]anthraquinones such as urdamycinone E, urdamycinone G, dehydroxyaquayamycin and urdamycin E also possess strong cytotoxic activities with IC_{50} value of 0.092 and 0.242 μg ml^{-1} against NCI-H187 cells, revealing a promising potential of C-glucosylated benz[α]anthraquinones as new lead compounds for antitumor. Dehydroxyaquayamycin B also showed an appreciable anti-fungal activity against *V. mali*, *C. orbiculare* and *F. graminearum*, and this was the first report of fungicidal activities of C-glycosylated benz[α]anthraquinones.

In conclusion, the natural product dehydroxyaquayamycin B is a new benz[α]anthracene glycoside that was isolated from the the broth of endophytic *S. blastomycetica* strain F4-20. Dehydroxyaquayamycin B showed considerable cytotoxic activities. The results presented in this paper highlighted endophytic actinomycetes as a rich source of bioactive compounds, and this was the first report of the secondary metabolites of *Streptomyces blastomycetica* genus.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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