Isofuranonaphthoquinone Produced by an *Actinoplanes* Isolate

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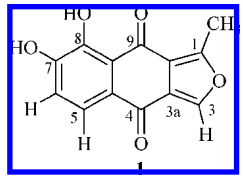
Received September 10, 2008

A new isofuranonaphthoquinone, 7,8-dihydroxy-1-methylnaphtho[2,3-*c*]furan-4,9-dione, was isolated from cultures of an *Actinoplanes* isolate obtained using an *in situ* diffusion technology that facilitates the isolation of soil microorganisms. This compound was demonstrated to have the ability to complex Fe(III). The structure was determined on the basis of spectroscopic data.

Only a few isofuranonaphthoquinone derivatives have been isolated from natural sources, and the majority are from plant and fungal sources.[1−10] A number of other compounds are structurally related to isofuranonaphthoquinone, including arthoniafurone B, which has one of the quinone carbonyl groups saturated.[5,8] The isofuranonaphthoquinone derivatives have been reported to have antioxidant and antiplasmodial activities.[9,10] Recently, these compounds were also demonstrated to be potent inhibitors of a mosquito glutathione transferase.[11] In this paper we describe the isolation and structure elucidation of a new isofuranonaphthoquinone, 7,8-dihydroxy-1-methylnaphtho[2,3-*c*]furan-4,9-dione (**1**), produced by an *Actinoplanes* strain that was isolated using our proprietary *in situ* diffusion chamber technology.[12]

The producing isolate ISO06811 was cultivated from a soil sample collected in the state of Colorado using our *in situ* diffusion technology.[13,15] This technology facilitates the isolation of filamentous microorganisms by permitting the thinner mycelia of filamentous bacteria to penetrate through the 0.2 μm pore size membrane into the agar insert. The constant contact of the agar insert with the soil provided continued access to the chemical background present in their native soil environment. The taxonomic identity of ISO06811 was determined by 16S rDNA sequence analysis. The 16S rDNA sequence (base pair 20 to base pair 710) of strain ISO06811 has been deposited in GenBank and has been given accession number FJ527243. The top sequence match as of December 2, 2008, using BLAST (nucleotide sequence comparison) to the GenBank database was 97% sequence similarity to *Actinoplanes* sp., strain 96 (GenBank accession no. AJ488567).



Compound **1** was identified during empirical fermentation-based screening of bacterial isolates for secondary metabolites of interest. HPLC analysis revealed that compound **1** was the major secondary metabolite when ISO06811 was cultured in a complex medium containing glucose, glycerol, salts, and complex protein.

The fermentation was scaled up to a 10 L volume to facilitate structure elucidation studies. Compound **1** was isolated from the fermentation mycelia by solvent extraction followed by solid-phase extraction and solvent trituration. The molecular formula was determined as $C_{13}H_8O_5$ on the basis of negative ion HRESIMS ($[M - H]^-$ *m/z* 243.0280, calcd for 243.0294) and NMR spectroscopic data (Table 1), indicating the presence of 10 double-bond equivalents. The UV spectrum shows a maximum absorption at 402 nm, suggesting the presence of a quinone moiety. The structure was elucidated by interpretation of 2D NMR spectra (COSY, HSQC, and HMBC) and comparison with reported isofuranonaphthoquinone data.[5−9] Two of the three aromatic protons are coupled to each other with a vicinal coupling constant of 8.4 Hz. These two proton signals were assigned to H-5 and H-6 on the basis of HMBC analysis. H-5 showed HMBC correlation to six carbons, including the two carbonyl carbons, while H-6 showed five correlations (Table 1). The phenolic protons showed all corresponding two- and three-bond correlations, as well as a four-bond correlation to the C-9 carbonyl carbon. The aromatic furan proton showed HMBC correlations to both C-4 and C-9 carbonyl carbons in addition to other correlations within the furan ring. The HMBC experiment also revealed correlations from the methyl proton to all the carbons of the furan ring and the C-9 carbonyl carbon. Because of the observation of HMBC correlations between proton signals of both aromatic rings and the carbonyl carbons, the quinone moiety must necessarily be placed in the central ring of the three-ring system to form an isofuranonaphthoquinone. Thus, the structure of compound **1** was determined to be 7,8-dihydroxy-1-methylnaphtho[2,3-*c*]furan-4,9-dione. This compound is closely related to the known 8-hydroxy-1-methylnaphtho[2,3-*c*]furan-4,9-dione and 5,8-dihydroxy-1-methylnaphtho[2,3-*c*]furan-4,9-dione isolated from plants.[5,7]

Table 1. NMR Data of **1** (DMSO-d_6, δ in ppm, *J* in Hz)

position	δ_C[a]	δ_H	HMBC[b]
1	160.3		
1-Me	13.7	2.70 (3H, s)	**1**, 3, 3a, 9, **9a**
3	144.6	8.48 (1H, s)	**1**, **3a**, 4, 9, **9a**
3a	122.8		
4	177.3		
4a	126.1		
5	120.6	7.57 (1H, d, 8.4)	**4**, 6, **7**, 8, **8a**, 9
6	120.3	7.17 (1H, d, 8.4)	4, **4a**, **7**, **8**, 8a
7	152.5		
8	151.3		
8a	117.9		
9	186.8		
9a	116.3		
7-OH		10.73 (1H, s)	**6**, 7, **8**
8-OH		12.88 (1H, s)	**7**, **8**, **8a**, 9

[a] Assignments supported by HSQC correlations. [b] Major correlations in bold.

Compound **1** was the most abundant component found in fermentations of this isolate. Although initially investigated for antibacterial activity, it failed to show any bioactivity against *Bacillus subtilis* 1A1 or *Escherichia coli* K12. Due to the fact that a characteristic $[2M - 2H^+ + Fe^{3+}]^+$ isotope pattern was observed during MS analysis, we

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10.1021/np8005716 CCC: $40.75
Published on Web 05/18/2009

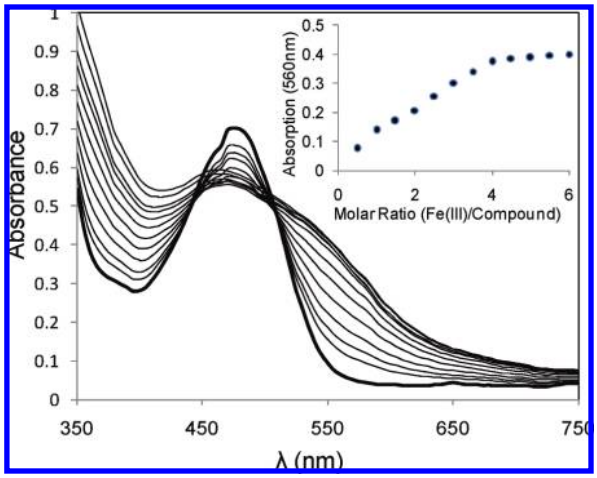

Figure 1. UV absorption spectra for **1** is altered by the addition of Fe(III). Inset shows the change in absorption at 560 nm as a function of Fe(III) addition.

postulated that compound **1** is a siderophore. To examine this, we titrated Fe(III) into a solution of compound **1** and observed an Fe(III)-dependent shift in the UV absorption spectrum (Figure 1). This shift continued in a linear manner until approximately 4 molar equiv of Fe(III) was added, at which point, the addition of Fe(III) had little effect on the UV absorption spectrum. These data reveal that compound **1** is able to complex Fe(III) at circumneutral pH and support our theory that it acts as a siderophore.

In summary, we have described a new member of the isofuranonaphthoquinone group of secondary metabolites and have characterized its ability to complex Fe(III). This is the first case of an isofuranonaphthoquinone derivative that has been isolated from a bacterial source. Furthermore, our data suggest that compound **1** is a new siderophore utilized by this *Actinoplanes* isolate to sequester Fe(III) to support growth.

Experimental Section

General Experimental Procedures. All NMR spectra were recorded on a Bruker-DRX-500 spectrometer equipped with a 5 mm QNP probe. ^{1}H, ^{13}C, COSY, HSQC, and HMBC NMR spectra were referenced to DMSO ($\delta_H = 2.51$, $\delta_C = 39.5$). High-resolution ESI-LC-MS data were recorded on a MicroMass Q-Tof-2 spectrometer equipped with an Agilent 1100 solvent delivery system and an online diode array detector using a Phenomenex Gemini-C18 reversed-phase column (50 × 2.0 mm, 3 μm particle size). Elution was performed with a linear gradient using deionized H_2O with 0.1% HCOOH and MeCN with 0.1% HCOOH as solvents A and B, respectively, at a flow rate of 0.2 mL/min. The gradient increased from 10% to 100% of solvent B over 20 min, with compound **1** eluting at 12.7 min. Analytical HPLC was performed on a Zorbax SB-C18 reversed-phase column (250 × 9.4 mm, 5 μm particle size) using a Shimadzu SCL-10AVP HPLC system including an SPD-M10AVP diode array detector set at 254 nm. Elution was performed with a linear gradient using deionized H_2O with 0.1% TFA and MeCN with 0.1% TFA as solvents A and B, respectively, at a flow rate of 4 mL/min. The gradient increased from 10% to 100% of solvent B over 30 min, with compound **1** eluting at 15.9 min.

Isolation of ISO06811. The *in situ* diffusion technology[13,15] involves gluing a 0.2 μm pore size polycarbonate membrane (47 mm diameter, Isopore, Millipore) to one end of a plastic O-ring (5.5 cm outer diameter, 3.3 inner diameter, 3 mm thick, Cat# SPS-2209-1375-0125, from WashersUSA.com), filling the insert with 3 mL of sterile 1% Bacto-agar supplemented with 1% vitamins (ATCC Cat# MD-VS), and sealing the other end with another semipermeable membrane (0.03 μm pore size polycarbonate membrane). The device was placed with the 0.2 μm membrane in direct contact with soil. After 2 weeks, the agar insert was removed to a sterile Petri plate and placed such that the side facing the soil was now exposed to air. After another 5 days of growth at room temperature, aerial hyphae were picked by sterile needles and purified by streaking them out onto 2% Bacto-agar plates. Isolates that grow well, such as ISO06811, were selected for further studies.

16S rDNA Sequence Analysis. 16S rDNA sequence analysis was utilized to determine the taxonomic identity of isolate ISO06811. Chromosomal DNA was isolated from approximately 10^6 cells after a 5 min vigorous agitation in the presence of 50 mg of glass beads and 100 μL of H_2O in a 0.5 mL Eppendorf tube. PCR amplification of nucleotide bases 20 through 710 of the gene encoding the 16S rDNA was carried out using ISO06811 chromosomal DNA, GoTaq Green Master Mix (Promega M7122), and universal primers Bac8F and 1492R.[14] PCR thermocycler parameters included 30 cycles of 95 °C for 30 s, 45 °C for 30 s, and 72 °C for 105 s. The amplified DNA fragment was sequenced by Macrogen (Rockville, MD) using primer 782R and compared by BLAST alignment to the GenBank nucleotide collection.

Fermentation. A colony of isolate ISO06811 was homogenized using a sterile pestle in a 1.5 mL Eppendorf tube containing 200 μL of sterile H_2O. The bacterial homogenate was transferred to a 250 mL Erlenmeyer flask containing 40 mL of seed broth (1.5% glucose, 1.0% malt extract, 1.0% soluble starch, 0.25% yeast extract, 0.5% casamino acids, and 0.005% $CaCl_2 \cdot 2H_2O$, pH adjusted to 7.0 before autoclaving). The seed broth was incubated for 7 days at 28 °C on a rotary shaker (1 in. throw, 200 rpm) prior to production medium inoculation at 2.5% (v/v). Production was performed in 500 mL aliquots of GGSC (2.0% glucose, 2.0% glycerol, 1.0% soy flour, 1.0% cottonseed embryo, 1.0% $CaCO_3$, and 0.1% $(NH_4)_2SO_4$) in 2 L tribaffled flasks for 7 days at 28 °C on a rotary shaker (2.5 in. throw, 200 rpm).

Isolation of Compound 1. The fermentation broth (10.5 L) was centrifuged at 10 000*g* for 30 min, and the pellet was extracted with MeOH (1.5 L). The MeOH extract was mixed with Diaion HP-20 resin (150 g), and the mixture was concentrated under reduced pressure. The resulting wet Diaion HP-20 resin was washed sequentially with 800 mL each of H_2O, 20% MeOH, 60% MeOH, and 100% MeOH. The 100% MeOH eluate was concentrated under reduced pressure to yield a dark solid. This mixture was triturated with EtOAc (2 × 50 mL), and the resulting EtOAc solution was concentrated to dryness under reduced pressure. The residue was triturated with MeOH (2 × 4 mL) to yield the insoluble yellow solid **1** (160 mg after high vacuum). Chromatographic separation was not necessary.

Biological Assays. Antibacterial activity was monitored by measuring the ability of compound **1** to inhibit growth of test strains. Exponentially growing test strains, *Bacillus subtilis* and *Escherichia coli,* diluted to $OD_{600} = 0.02$ in Mueller Hinton broth were evenly applied as a thin layer on the surface of a Petri plate of Mueller Hinton agar (about 0.1 mL of cells to a surface area of 100 cm^2). After the surface was dried, a 5 μL aliquot of compound **1** (in 50% DMSO), starting from 5 mg/mL, was spotted onto the surface of the plate. After 20 h of incubation at 37 °C, the diameter of zones of inhibition was measured.

Fe(III)-Dependent UV Absorption. A 50 μM solution of **1** was made in 5 mM HEPES buffer (pH 7.0) at 20 °C. Fe(III) was removed from the solution by the addition of Chelex 100 resin (~5% v/v) (Sigma-Aldrich) with subsequent centrifugation and removal of the Fe-free solution to a quartz cuvette. UV absorption spectra were recorded (350−750 nm, 1 cm slit) using a SpectraMax Plus UV spectrometer (Molecular Devices) in a 1 mL quartz cuvette with a 1 cm path length and 20 nm slit width. $Fe_2(SO_4)_3$ was added from a 10 mM stock solution to achieve 25 μM stepwise increases in Fe(III). The solution was mixed after Fe(III) addition and allowed to sit at room temperature for 5 min prior to UV absorption measurement.

Acknowledgment. This work was supported by grants R44 AI063616-03 and 1R43AI78572-01 from the National Institutes of Health.

Supporting Information Available: UV spectrum, LC-MS chromatograms, HRESIMS, and ^{1}H, ^{13}C, COSY, HSQC, and HMBC NMR spectra of compound **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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NP8005716