Notes

Antibiotic Bisanthraquinones Produced by a Streptomycete Isolated from a Cyanobacterium Associated with *Ecteinascidia turbinata*

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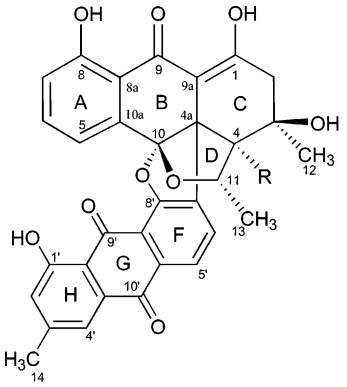
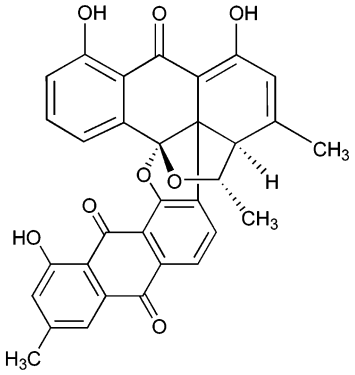
Chemical studies of a streptomycete isolated from a cyanobacterium associated with the tropical tunicate *Ecteinascidia turbinata* led to the bioassay-guided purification of two antibacterial bisanthraquinone metabolites and a cytotoxic artifact. The structures, including relative configurations of these octacyclic compounds, were established by spectroscopic analyses. Their potent antibacterial properties (IC_{50} = 0.15−130 μM) versus methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecalis* and cytotoxic effects against HCT-116 cells are presented.

The ascidian *Ecteinascidia turbinata* produces the powerful anticancer agent ET743.[1] The striking similarity of ET743 to the saframycin antibiotics produced by actinomycetes[2] raises speculation that the compound may be of microbial origin. Interest in the true source of ET743 is especially keen due to the trace quantities obtained from the rather rare tunicate.[1] We detected strong antimicrobial and cytotoxic activity in the culture medium of a *Streptomyces* sp. isolated from a cyanobacterium found in association with *E. turbinata* collected in Puerto Rico. Further investigation revealed that the active principles were not ET743 or saframycin analogues, but rather a series of unusual bisanthraquinones. This paper reports the investigation of these bacterial metabolites.

Bacterial strain # N1-78-1 was isolated from the cultured cells of an unidentified unicellular blue-green alga, URI strain # N36-11-10, which was isolated from the ascidian *Ecteinascidia turbinata*, collected at La Parguera, Puerto Rico. N1-78-1 was identified by16S rRNA gene sequence comparison as belonging to the genus *Streptomyces*. The actinomycete was cultivated in 28 1-L shake flasks at 31 °C in a seawater medium that included chitin, glycerol, and L-asparagine. After 12 days, the whole cultures were extracted with ethyl acetate, and bioassay-guided fractionation of the crude extract by LH-20 column chromatography and C18 HPLC yielded pure compounds **1** (50 mg) and **2** (15 mg) as yellow, amorphous solids. HRESIMS of **1** gave a pseudomolecular ion $[M + H]^+$ at *m*/*z* 553.1506, appropriate for a molecular formula of $C_{32}H_{24}O_9$. This formula, in conjunction with ^1H and ^{13}C NMR data, suggested that **1** likely matched the planar structure of BE-43472B, a streptomycete metabolite claimed in a Japanese patent.[3] However, since the patent literature provided neither details related to the structure elucidation nor the relative stereochemistry of the five chiral centers, a closer examination of the spectroscopic evidence for the structure of **1** was undertaken.



1 (R=H)
2 (R=OH)



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Analysis of 1D and 2D NMR spectra (Table 1) established two major substructures that accounted for 20 of the 21 degrees of unsaturation required by the molecular formula. Inspection of ^{13}C, DEPT, and HMQC NMR data indicated that metabolite **1** contained nine methine, one methylene, three methyl, and 19 quarternary carbons. A COSY spin system comprising an oxygenated methine (H-11, δ 4.92) coupled to both a methine proton (H-4, δ 2.39) and a methyl group (H-13, δ 1.49) revealed partial connectivity through the D ring. Figure 1 shows selected two- and three-bond HMBC correlations that further defined the two substructures. ^{13}C NMR chemical shifts of carbons comprising the F−H rings closely match reported values for the anthraquinone chrysophanol.[4] A key HMBC correlation from H-4 to C-7′ provided the only through-bond correlation between the upper and lower ring assemblies. Since Figure 1 accounts for all of the atoms in the molecular formula with one additional oxygen, C-8′ must be connected to C-10 through

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Table 1. NMR Spectral Data[a] for Compound **1** in DMSO-d_6

pos	δ_C	δ_H (mult, J in Hz)	COSY	HMBC
1	182.3			
2α	44.8 (CH_2)	3.03 (d, 16)	2β	1, 3, 4, 9a
2β		2.59 (d, 16)	2α	1, 3, 4, 9a, 12
3	70.2			
4	64.6 (CH)	2.39 (d, 5.1)	11	4a, 7′, 9a, 11, 12, 13
4a	55.9			
5	118.6 (CH)	7.40 (d, 7.6)	6, 7	7, 8a, 10
6	137.2 (CH)	7.71 (m)	5, 7	8, 10a
7	118.5 (CH)	6.97 (d, 8.2)	5, 6	5, 8, 8a
8	160.6			
8a	112.7			
9	186.6			
9a	102.2			
10	121.1			
10a	137.3			
11	80.5 (CH)	4.92 (m)	4, 13	3, 4, 4a, 10
12	29.4 (CH_3)	1.27 (s)		2, 3, 4
13	24.7 (CH_3)	1.49 (d, 6.5)	11	4, 11
14	21.5 (CH_3)	2.38 (s)		3′, 4′
1′	161.5			
2′	123.8 (CH)	7.14 (bs)	4′	1′, 4′, 9a′, 14
3′	147.9			
4′	119.7 (CH)	7.44 (bs)	2′	2′, 9a′, 10′, 14
4a′	132.5			
5′	121.1 (CH)	7.70 (d, 8)	6′	7′, 8a′
6′	129.4 (CH)	7.66 (d, 8)	5′	8′, 10a′
7′	144.2			
8′	155.4			
8a′	116.2			
9′	187.0			
9a′	114.2			
10′	181.3			
10a′	133.4			
OH-1		14.46 (s)		
OH-3		5.18 (s)		3, 4, 12
OH-8		12.05 (s)		7, 8, 8a
OH-1′		12.60 (s)		1′, 2′, 9a′

[a] Data were obtained at 100 MHz (^{13}C) and 400 MHz (^1H) with chemical shifts (δ) in ppm.

an ether linkage, thus forming the E ring and accounting for the one remaining degree of unsaturation.

Attempts to prepare crystals suitable for X-ray analysis were unsuccessful, and therefore the relative stereochemistry of **1** was deduced via NOE-difference experiments (Figure 2a). Irradiation of the H-13 methyl protons showed enhancement of the H-4 methine, indicating their *cis* arrangement on the D ring. A second NOE signal to the H-4 methine proton was observed from the H-12 methyl group. Irradiation of the C-3 hydroxyl proton showed an enhancement to the H-11 methine, thus establishing the relative stereochemistry of the methyl and hydroxyl substituents on C-3. The orientation of the fused E−H rings relative to the A−D rings was deduced by observing a minor NOE enhancement from the H-6′ methine to the H-12 methyl protons. Molecular modeling predictions (Figure 2b; Cambridge Soft CHEMDRAW 3-D; 2.3−

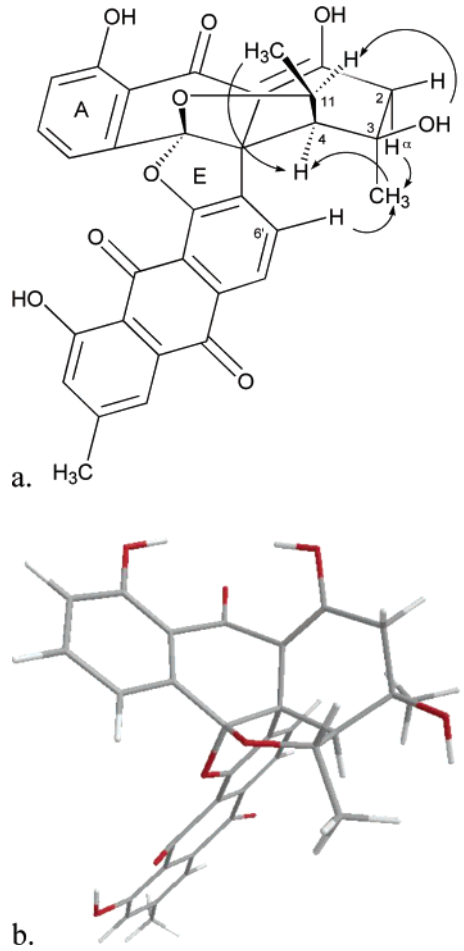

Figure 2. (a) Relative stereochemistry of **1** with selected NOE correlations. (b) Three-dimensional representation of **1** after MM2 energy minimization (Cambridge Soft CHEMDRAW 3-D).

3.5 Å) provided acceptable internuclear distances[5] of all observed NOE correlations. A very minor enhancement between the C-12 methyl and H-11 methine was also observed and may be accounted for by chair−chair flipping of the substituted cyclohexene C ring.

Compound **2** was isolated as a yellow, amorphous solid with HRESIMS $[M + H]^+$ of 569.1450, consistent with a molecular formula of $C_{32}H_{24}O_{10}$. Both UV and IR spectra of this compound closely resembled that of **1** with the most notable difference being a more intense IR hydroxyl absorption at 3477 cm^{-1}. NMR experiments with **2** were conducted in $CDCl_3$ (Table 2) due to degradation of the metabolite in DMSO. While the overall number of carbon atoms of **1** and **2** remained the same, ^{13}C and DEPT NMR experiments indicated the replacement of the C-4 methine with an oxygenated quaternary sp^3 carbon at δ 84.7. A strong three-

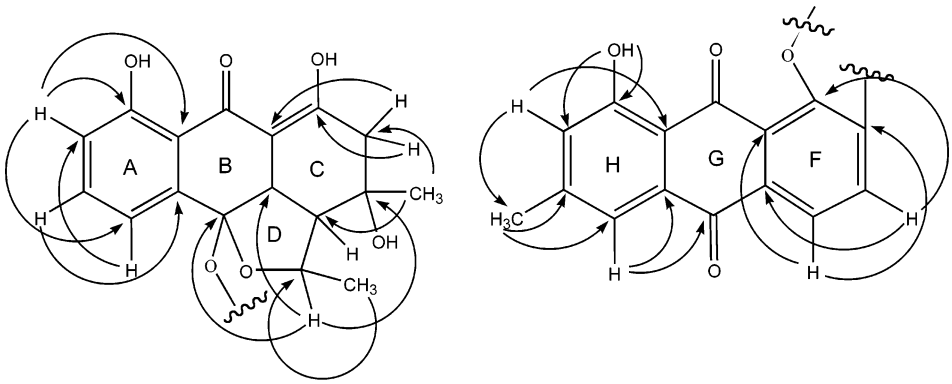

Figure 1. Selected HMBC correlations of upper and lower anthraquinone ring assemblies.

Table 2. ^{13}C and ^{1}H NMR[a] Spectroscopic Data for Compounds **2** and **3**

	2[b]		**3**[c]	
pos	δ_C	δ_H	δ_C	δ_H
1	175.7		172.1	
2	42.2 (CH_2)	$\alpha = 3.01$ (d, 18) $\beta = 2.72$ (d, 18)	120.4 (CH)	6.37 (s)
3	73.1		119.8	
4	84.7		57.5 (CH)	2.94 (d, 10.2)
4a	60.5		54.3	
5	119.3 (CH)	7.47 (dd, 1.2, 7.6)	118.7 (CH)	7.41 (d, 7.8)
6	137.8 (CH)	7.55 (m)	137.2 (CH)	7.66 (m)
7	118.5 (CH)	6.91 (dd, 1.2, 8.4)	118.1 (CH)	6.97 (d, 8.4)
8	161.8		160.7	
8a	113.4		113.3	
9	186.9		186.5	
9a	101.9		93.9	
10	120.0		119.9	
10a	137.5		137.5	
11	84.2 (CH)	5.06 (m, 6.6)	84.5 (CH)	4.52 (m)
12	25.6 (CH_3)	1.38 (s)	23.5 (CH_3)	2.03 (s)
13	17.0 (CH_3)	1.57 (d, 6.6)	20.9 (CH_3)	1.55 (d, 5.7)
14	22.2 (CH_3)	2.41 (s)	21.5 (CH_3)	2.39 (s)
1′	162.6		161.6	
2′	124.4 (CH)	7.05 (m, 0.8)	123.8 (CH)	7.16 (s)
3′	148.2		148.1	
4′	120.6 (CH)	7.55 (m)	119.9 (CH)	7.44 (bs)
4a′	132.6		132.5	
5′	121.7 (CH)	7.87 (d, 7.6)	121.3 (CH)	7.69 (m)
6′	130.7 (CH)	7.41 (d, 7.6)	128.5 (CH)	7.52 (d, 7.4)
7′	138.1		140.5	
8′	157.6		155.1	
8a′	116.9		116.7	
9′	190.4		187.6	
9a′	114.4		114.1	
10′	181.9		181.2	
10a′	134.8		134.1	
OH-1		13.87 (s)		14.35 (s)
OH-3		2.08 (s)[d]		
OH-4		2.60 (s)[d]		
OH-1′		12.65 (s)		12.64 (s)

[a] Data were obtained at 100 MHz (^{13}C) and 400 MHz (^{1}H) with chemical shifts (δ) in ppm. [b] Recorded in $CDCl_3$. [c] Recorded in DMSO-d_6. [d] Unable to distinguish between resonances due to a lack of definitive HMBC correlations.

bond HMBC correlation was observed from the C-13 methyl to new quaternary C-4. The ^{1}H NMR spectrum of **2** differed from **1** by substitution of the H-4 resonance with an exchangeable proton peak. In the ^{1}H and COSY spectra, H-11 was coupled only to the H-13 methyl group. All of these data are consistent with metabolite **2** as the C-4 alcohol analogue of **1** and matching the planar structure of BE-43472A.[3] The relative stereochemistry of **2** is assumed to match that of **1**.

During the course of this investigation, it became apparent that bisanthraquinones **1** and **2** were degrading to single artifacts during long NMR acquisitions in DMSO-d_6. Partial conversion of **1** to **3** was observed by allowing **1** to stand in DMSO at ambient temperature for several days. Reversed-phase HPLC demonstrated that the product was slightly less polar. HRESIMS of **3** gave a pseudomolecular ion $[M + H]^+$ at *m*/*z* 535.1401, appropriate for a molecular formula of a $C_{32}H_{22}O_8$ and suggesting elimination of water from the parent molecule. The elimination hypothesis was confirmed by NMR analysis. Significant downfield chemical shifts were observed for C-2 (δ 120.4) and C-3 (δ 119.8), indicating the site of elimination. In the ^{1}H spectrum, the diastereotopic methylene group of **1** was replaced with an olefinic H-2 proton appearing at δ 6.37. A COSY correlation between this newly formed olefin resonance and the H-12 methyl group was assigned to allylic coupling. The COSY spin system through H-13, H-11, and H-4 was still intact. The above data are consistent with bisanthraquinone **3** being the $\Delta^{2,3}$ olefin derivative of the parent compound **1**.

Table 3. Biological Activities of Bisanthraquinone Derivatives

compound	MRSA[a]	VRE[a]	HCT-116[a]
1	0.15	2.0	3.3
2	0.36	12	5.6
3	31	130	6.6

[a] IC_{50}, μM.

The bacterial metabolites **1** and **2** potently inhibited the growth of methicillin-resistant *Staphylococcus aureus* (MRSA, ATCC 43300), but were greater than 10-fold less effective against vancomycin-resistant *Enterococcus faecalis* (VRE, ATCC 51299). Compound **3**, the dehydration product of **1**, displayed a pronounced 220-fold decrease in inhibition of MRSA versus the parent compound, indicating a requirement for either ring flexibility or the C-3 hydroxyl group for maximum potency. All three bisanthraquinone derivatives were moderately active against HCT-116 human colon tumor cells (Table 3). Metabolites **1** and **2** are also reported to possess comparable cytotoxic activities against several different cell lines.[3]

The blue-green alga and associated streptomycete are of intriguing origin. The tunic surface of *E. turbinata* is known to be colonized by both epizoic bacteria and microalgae,[6] and it is likely that the cyanobacterium investigated here was an epibiont. It is not evident from this study whether the isolated organisms have any widespread or specific association with *E. turbinata*. Remarkably, the streptomycete was separated from an algal culture that had been maintained for two years under strict sterile conditions. To our knowledge this has never before been reported and is especially intriguing given the structural similarities between some cyanobacterial and streptomycete metabolites.[7−9] Only very weak cytotoxicity was observed with extracts derived from cultures of the alga itself (Shimizu, unpublished results). Bisanthraquinones from actinomycetes are rare, with the julimycins and julichromes being the only other examples reported to date.[10,11] The bisanthraquinones reported here are uniquely fused via a furan ring that fixes the molecule in a fairly rigid three-dimensional conformation. Given the sensitivity of the antibacterial activity to elimination of the C-3 hydroxyl group, we are currently investigating further structure−activity relationships of these antibiotics.

Experimental Section

General Experimental Procedures. Optical rotations were measured on an Autopol III automatic polarimeter (Rudolf Research), UV spectra were recorded on a DU 800 spectrophotometer (Beckmann-Coulter), and IR spectra were acquired on a Nexus 470 FT-IR (Thermo Nicolet). NMR spectra were recorded on a Bruker Biospin spectrometer (400 MHz for ^{1}H, 100 MHz for ^{13}C) and were referenced to residual solvent signals with resonances at $\delta_{H/C}$ 7.24/77.0 ($CDCl_3$) and $\delta_{H/C}$ 2.49/39.5 (DMSO-d_6). NOE difference experiments with **1** were conducted in DMSO-d_6 (9 mg in 650 μL). High-resolution ESI mass spectrometry was accomplished in 50:50 acetonitrile/water (+ 0.1% formic acid) on a Micromass Q-Tof Ultima instrument at the University of Illinois Urbana−Champaign Noyes Laboratory. HPLC was performed using a Waters 600 pump and a 486 tunable absorbance detector.

Isolation and Fermentation. The *Streptomyces* strain # N1-78-1 was isolated from the cultured cells of an unidentified unicellular blue-green alga (cyanobacterium), URI strain # N36-11-10, which had been isolated from the ascidian *Ecteinascidia turbinata*, collected at La Parguera, Puerto Rico. The alga was isolated as a single colony from an *E. turbinata* tunic spread onto a seawater agar growth medium. The cultured algal cells grown in the F/2-Si medium (23 °C, 16 h light:8 h dark cycle, static) were spread onto a plate of YMA medium (4 g of glucose, 4 g of yeast extract, 10 g of malt extract, 20 g of bactoagar, and 1 L of distilled water) supplemented with cycloheximide (50 μg/mL). Bacterial colonies that formed were further selected by reinoculation on the same plates. Strict sterile technique was maintained throughout the process of algal isolation and laboratory cultivation. The isolated bacterial strain appeared as round, leathery colonies and formed grayish aerial mycelia characteristic of *Streptomyces.* Large-

scale cultivation for chemical studies was undertaken in 28 × 1 L shake flasks for 12 days at 31 °C using a marine medium containing 4 g of glycerol, 0.4 g of L-asparagine, and 2 g of chitin per liter of seawater.

Phylogenetic identification of N1-78-1 was accomplished using 16S rRNA gene sequence comparison. A colony was macerated and suspended in 10 μL of Lyse-n-Go PCR reagent (Pierce, Rockford, IL) to extract DNA. The Lyse-n-Go lysate (1 μL) was combined with 20 μL of molecular biology grade water and 2 μL of a 10 μM stock of the forward and reverse primers[12] and added to PureTaq Ready-To-Go PCR beads (Amersham Biosciences, Piscataway, NJ). The amplification reaction was carried out in a ThermoHybaid PCR Sprint thermal cycler. The PCR product was purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA), quantified with a Pharmacia Biotechnology Ultra 4000 spectrophotometer, and sequenced using a Beckman Coulter CEQ 8000 sequencer (URI Genomics Center). A BLAST search (NCBI, 5-2-2006) revealed that the amplified 16S rRNA gene sequence (370 bases, deposited with GenBank as Assession No. DQ470014) clearly places this isolate in the genus *Streptomyces*, with the nearest characterized species being *Streptomyces melanosporofaciens* (97% similarity).

Extraction and Purification. Ethyl acetate extraction of the spent culture broths resulted in bright yellow extracts, which were subsequently filtered through Celite, dried over Na_2SO_4, and concentrated in vacuo (505.8 mg). Bioassay-guided fractionation commenced with Sephadex LH20 (Fluka) column chromatography (3:2:1 isooctane/methanol/toluene, 2.6 × 67.5 cm column, 4 mL fractions). The antibacterial activity, as well as the bright yellow color, was concentrated in fractions 40−94. These were combined (86 mg) and further purified by LH-20 (100% methanol, 4 × 100 cm column) to provide **1** and **2** in nearly pure form. Reversed-phase HPLC (19 × 100 mm Waters X-Terra RP18 column, 10 mL/min, 20−70% acetonitrile in H_2O over 120 min) of these two fractions yielded the pure metabolites **1** (50 mg) and **2** (15 mg). Artifact **3** was primarily acquired via HPLC purification of an NMR sample of **1** in DMSO-d_6 using the above conditions.

Bisanthraquinone derivative 1: yellow amorphous solid; $[\alpha]^{22}_D$ +650 (*c* 1.0, DMSO); IR ν_{max} 3418, 2970, 1668, 1613, 1579, 1452, 1331, 1266, 1235, 1205, 1125, 958, 816, 801, 747, 714 cm^{-1}; UV (MeOH) λ_{max} (log ϵ) 229 (4.56), 261 (4.31), 287 (3.93), 361 (4.06), 415 (3.94) nm; HRESIMS $[M + H]^+ = 553.1506$ (553.1499 calcd for $C_{32}H_{25}O_9^+$).

Bisanthraquinone derivative 2: yellow amorphous solid; $[\alpha]^{23}_D$ +538 (*c* 0.23, $CHCl_3$); IR ν_{max} 3477, 2978, 1663, 1610, 1573, 1454, 1426, 1266, 1205, 1168, 952, 809, 743 cm^{-1}; UV (MeOH) λ_{max} (log ϵ) 229 (4.59), 261 (4.35), 287 (3.97), 363 (4.08), 416 (3.98) nm; HRESIMS $[M + H]^+ = 569.1450$ (569.1448 calcd for $C_{32}H_{25}O_{10}^+$).

Bisanthraquinone derivative 3: yellow amorphous solid; $[\alpha]^{23}_D$ +446 (*c* 0.37, $CHCl_3$); IR ν_{max} 2921, 1708, 1671, 1634, 1563, 1453, 1429, 1365, 1263, 1235, 954, 905, 849, 817, 749, 670 cm^{-1}; UV (MeOH) λ_{max} (log ϵ) 226 (4.77), 257 (4.58), 285 (4.22), 392 (4.46) nm; HRESIMS $[M + H]^+ = 535.1401$ (535.1393 calcd for $C_{32}H_{23}O_8^+$).

Antimicrobial Bioassay. Antibacterial broth dilution assays[13] were conducted using MRSA (ATCC 43300, trypticase soy media) and VRE (ATCC 51299, brain heart infusion media). MRSA and VRE were grown for 14 h in 10 mL tubes on a rotary shaker at 38 °C. Cultures were diluted with sterile medium to achieve an optical absorbance of 0.04−0.06 at 600 nm and then further diluted 10-fold before distributing into 96-well microtiter plates. Ten replicates of each compound were tested in dilution series, and results were measured after 18 h by measuring optical absorbance at 600 nm. Tetracycline was used as a standard and provided consistent IC_{50} values of 0.2 μg/mL (MRSA) and 0.7 μg/mL (VRE).

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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