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New bisanthraquinone antibiotics and semi-synthetic derivatives with potent activity against clinical *Staphylococcus aureus* and *Enterococcus faecium* isolates

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Abstract—The escalation of antibiotic resistance among Gram-positive pathogens presents increasing treatment challenges and requires the development of innovative therapeutic agents. Here, we present the antimicrobial properties of structurally unusual bisanthraquinone metabolites produced by a marine streptomycete and four semi-synthetic derivatives. Biological activities were measured against clinically derived isolates of vancomycin-resistant *Enterococcus faecium* (VRE), and methicillin-susceptible, methicillin-resistant, and tetracycline-resistant *Staphylococcus aureus* (MSSA, MRSA, and TRSA, respectively). The most potent antibiotic displayed MIC_{50} values of 0.11, 0.23, and 0.90 μM against a panel ($n = 25$ each) of clinical MSSA, MRSA, and VRE, respectively, and was determined to be bactericidal by time-kill analysis.

1. Introduction

Infections due to antibiotic resistant bacteria, especially staphylococci and enterococci, present increasing treatment challenges for clinicians and result in heightened morbidity and mortality. In 2002, the frequency of methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-resistant *Streptococcus epidermis* (MRSE), and vancomycin-resistant *Enterococcus* sp. (VRE) recorded in intensive care units was 57.1%, 89.1%, and 27.5%, respectively.[1] Nosocomial MRSA infections commonly have reduced susceptibility to classes of antibiotics other than the β-lactams, including macrolide, fluoroquinolone, aminoglycoside, tetracycline, and lincosamide antibiotics.[1] Of particular concern has been the identification of glycopeptide resistant strains of *S. aureus*, since vancomycin is often an antibiotic of last resort for hospital cases. Furthermore, the rising incidence of community-associated MRSA is alarming since infections have occurred in otherwise healthy individuals with no known health risks.[2] There is a critical need for new therapeutics to combat these evolving pathogens.

Marine actinomycetes have recently attracted attention as an emerging resource for the discovery of new antibiotics.[3–5] Unlike their terrestrial relatives, marine actinomycetes have yet to be extensively investigated. Moreover, genetic adaptations to the marine environment appear to include novel biosynthetic capabilities.[6,7] Given the remarkable success in developing clinically useful drugs from terrestrial actinomycetes, future investigations of marine strains hold tremendous promise.

During a recent investigation of a marine streptomycete isolated from a cyanobacterium collected in Puerto Rico, we identified two unusual bisanthraquinones (**1** and **2**) with strong antimicrobial activities against ATCC strains of MRSA and VRE.[8] Assignment of their relative stereochemistry revealed that these likely represent a new structural class among known antimicrobial agents. Biological evaluation of a serendipitous dehydration artifact (**5**) indicated that modifications to the C-ring could profoundly alter the antibiotic properties.

Keywords: Antibiotics; Marine streptomycete; Semi-synthesis; Bisanthraquinone.
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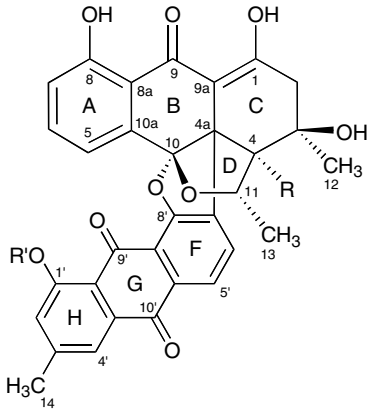
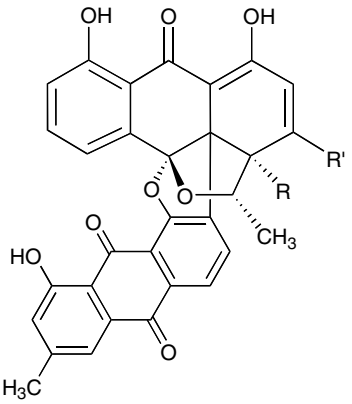


1: R=H, R'=H
2: R=OH, R'=H
3: R=H, R'=CH_3
4: R=OH, R'=CH_3



5: R=H, R'=CH_3
6: R=OH, R'=CH_3
7: R=H, R'=COH
8: R=H, R'=CH_2OH

This intriguing result led us to further investigate how structural variations in the C-ring can modulate the antibacterial properties and therapeutic index.

Here, we present new semi-synthetic bisanthraquinones and additional natural products that further define structure–activity relationships in this series. We also present a detailed evaluation of antibiotic properties versus a panel of drug resistant Gram-positive pathogens collected from patients admitted to the Veterans Affairs Medical Center in Providence, Rhode Island. Time-kill studies were used to determine bactericidal activity of the most potent analogues against clinical MSSA, MRSA, and VRE isolates.

2. Results

2.1. Isolation and characterization of bisanthraquinone metabolites

Bisanthraquinone metabolites **1** and **2**, also known respectively as BE-43472B and BE-43472A,[9] were isolated as previously described from a 28-L culture of a marine-derived *Streptomyces* sp. (URI strain No. N1-78-1, GenBank Accession No. DQ470014).[8] Reexamination of the ethyl acetate extract of the whole culture broths by analytical C18 HPLC revealed the presence of two minor and related metabolites. Purification of these by size exclusion (Sephadex LH-20) and silica-gel chromatography followed by reversed-phase HPLC yielded 2.5 mg each of metabolites **3** and **4**. High-resolution ESI mass spectrometry measurements gave $[M+H]^+$ pseudomolecular ions appropriate for molecular formulas of $C_{33}H_{26}O_9$ (**3**) and $C_{33}H_{26}O_{10}$ (**4**). The ^1H NMR spectrum of **3** ($CDCl_3$) was remarkably similar to that of **1**, with the distinguishing features being a new methyl singlet at δ 4.00 and the absence of the H-1′ phenolic hydroxyl resonance at δ 12.60. A ^{13}C methyl signal at δ 56.4 in the DEPT-135 spectrum was consistent with the introduction of an aromatic methoxyl substituent. Careful comparison of ^1H and ^{13}C NMR data revealed that the structure of metabolite **3** is identical to BE-43472D, the C-1′ methoxyl derivative of **1**.[9] Identical ^1H and ^{13}C chemical shifts through the A–E rings indicate that the relative stereochemistry matches that previously determined for **1**.[8] Similar NMR spectroscopic analysis and the mass data showed that metabolite **4** matched BE-43472C, the C-1′ methoxyl derivative of **2**. The ^1H NMR assignments for these two metabolites are presented here for the first time (Table 1).

2.2. Semi-synthesis of bisanthraquinone analogues

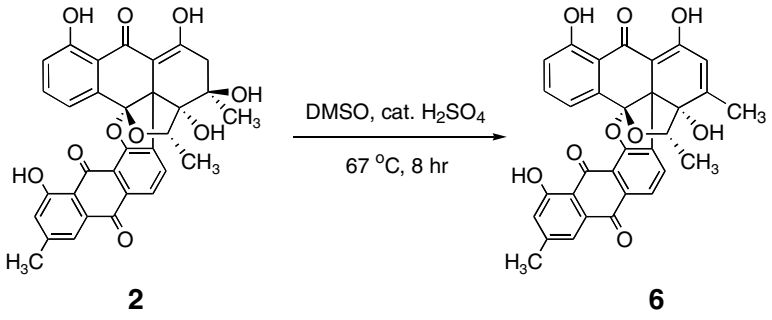
Bisanthraquinone metabolite **1** was previously found to dehydrate in DMSO at room temperature to yield artifact **5**.[8] The antibacterial potency of this elimination product diminished significantly, being 206- and 65-fold weaker versus MRSA (ATCC 43300) and VRE (ATCC 51299), respectively. It was not clear if the decreased activity was due to conformational changes induced by the C-ring olefin, the loss of a hydrogen bonding substituent, or contributions from both. To help clarify this issue, a series of semi-synthetic products were prepared. Bisanthraquinone **2** contains vicinal alcohols at the C-3 and C-4 positions, and therefore would retain a hydrogen bonding substituent proximal to the site of dehydration. Sulfuric acid-catalyzed dehydration of **2** in DMSO at 67 °C provided the $\Delta^{2,3}$ olefin derivative **6** in 87% yield (Scheme 1).

In the course of generating additional **5**, an increase in the reaction temperature to 75 °C resulted in the formation of both **5** and a second, more polar compound **7**. Following purification by reversed-phase HPLC, the structure of the new product was deduced by spectroscopic analysis. HRESIMS of **7** gave a pseudomolecular ion $[M+H]^+$ at *m/z* 549.1188, appropriate for a molecular formula of $C_{32}H_{21}O_9$. New IR absorbances observed at 1585, 3064, and 3550 cm^{-1} suggested the introduction of a conjugated aldehyde. In the ^1H NMR spectrum, the presence of a C-2 olefinic proton at δ 7.09 revealed that

Table 1. [1]H NMR assignments[a] for bisanthraquinone metabolites **3** and **4**, and derivatives **6**–**7**

Position	**3** δ_H (m, *J* in Hz)	**4** δ_H (m, *J* in Hz)	**6** δ_H (m, *J* in Hz)	**7** δ_H (m, *J* in Hz)	**8** δ_H (m, *J* in Hz)
2a	2.82 (d, 16)	2.75 (d, 18)	6.17 (s)	7.09 (s)	6.5 (s)
2b	2.71 (d, 16)	2.98 (d, 18)	NA	NA	NA
4	2.39 (d, 5.2)	NA	NA	3.39 (d, 10)	2.77 (d, 10)
5	7.62 (m)	7.49 (m)	7.51 (m)	7.59 (m)	7.54 (m)
6	7.52 (dd, 8.0)	7.48 (m)	7.54 (m)	7.61 (m)	7.55 (m)
7	6.89 (d, 8.4)	6.85 (m)	6.92 (d, 7.6)	6.95 (m)	6.93 (m)
11	4.96 (m)	5.07 (m)	4.77 (m)	4.46 (m)	4.56 (m)
12a	1.37 (s)	1.35 (s)	2.00 (d, 1.2)	9.72 (s)	4.34 (d, 18)
12b	NA	NA	NA	NA	4.19 (d, 18)
13	1.61 (d, 6.4)	1.63 (d, 6.0)	1.48 (d, 6.4)	1.57 (d, 6.0)	1.61 (d, 6.4)
14	2.45 (s)	2.44 (s)	2.40 (s)	2.40 (s)	2.41 (s)
15	4.00 (s)	3.97 (s)	NA	NA	NA
2′	7.08 (s)	7.05 (s)	7.03 (s)	7.05 (s)	7.07 (s)
4′	7.65 (m)	7.61 (s)	7.56 (m)	7.54 (s)	7.54 (m)
5′	7.78 (d, 7.6)	7.74 (d, 8.0)	7.88 (d, 7.6)	7.80 (d, 8.0)	7.83 (d, 7.6)
6′	7.25 (d, 7.8)	7.32 (d, 8.0)	7.41 (d, 7.6)	7.27 (d, 8.0)	7.36 (d, 7.6)
OH-1	14.42 (s)	13.84 (s)	14.43	13.7 (s)	14.36
OH-8	11.95 (s)	11.86 (s)	12.03	11.89 (s)	12.07
OH-1′	NA	NA	12.66 (s)	12.71 (s)	12.76

NA, not applicable.
[a] Spectra recorded in $CDCl_3$ at 400 MHz.



Scheme 1. Dehydration of diol **2** to form semi-synthetic product **6**.

dehydration had still occurred. However, the allylic methyl signal of **5** at δ 2.03 ppm was absent, and a new singlet resonance at δ 9.72 was prominent. A DEPT-135 experiment showed a methine ^{13}C resonance at δ 190.6, further supporting the installation of a conjugated aldehyde. A two-bond HMBC correlation from the aldehydic proton to C-3 and three-bond correlations from H-2 and H-4 to the new carbonyl at δ 190.6 confirmed that **7** was the C-12 oxidation product of **5** (Fig. 1).

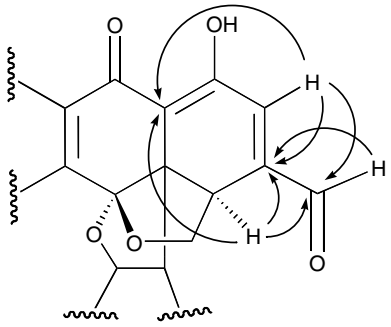
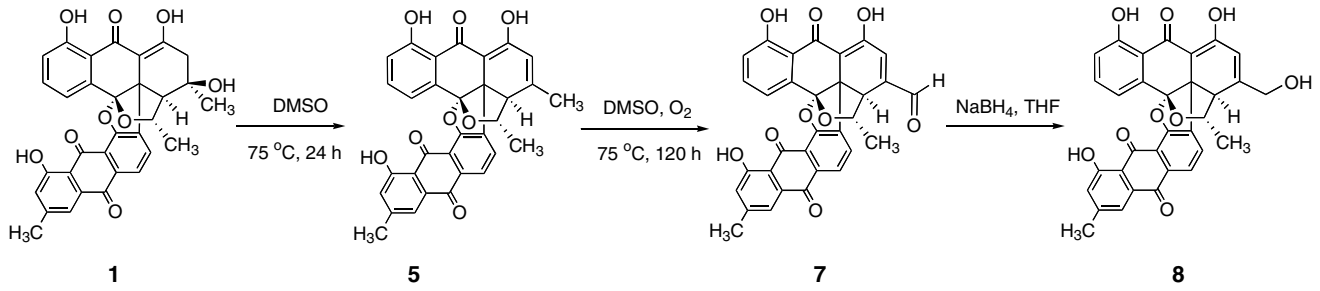


Figure 1. Selected HMBC correlations observed for **7**.

Transformation of **1** to **7** in DMSO appears to proceed through the allylic methyl intermediate **5** via an autoxidation mechanism (Scheme 2). In reactions conducted with purified **5** at 75 °C under an O_2 atmosphere, complete consumption of the starting material was observed after 124 h (66% yield). A series of small-scale experiments (0.2 mg of **5** in 1 mL DMSO) revealed that the reaction is accelerated by periodic sparging with oxygen and can be catalyzed by the addition of dimethylaminopyridine (Table 2). Conversely, reactions sparged with argon did not proceed, and the presence of 0.1 equiv of ethylenediaminetetraacetic acid (EDTA) quenched the oxidation. The addition of 0.1 equiv deferoxamine, an iron chelating reagent, had no effect on the reaction, nor did autoxidations conducted in the dark. Increases in temperature beyond 75 °C resulted in reduced yields of **7** (data not shown). The aldehyde was readily reduced with 1:1 $NaBH_4$ in anhydrous THF to provide the primary alcohol derivative **8**.

2.3. Biological activities of bisanthraquinone analogues

Clinical isolates of Gram-positive bacteria were obtained by random samplings from patients at the



Scheme 2. Dehydration, autoxidation, and reduction of bisanthraquinone metabolite **1** to produce semi-synthetic derivatives **5**, **7**, and **8**.

Table 2. Reaction conditions and yields for the autoxidation of **5** to aldehyde **7**

Solvent, reagents	Atmosphere	Reaction time (h)/temp (°C)	% yield of aldehyde **7**
DMSO	O_2	124/75	66[a]
DMSO, dark	O_2	18/75	25
DMSO	O_2	18/75	27
DMSO	Air	18/75	7
DMSO	Argon	18/75	1
DMSO, 1 equiv DMAP	Air	18/75	30[b]
DMF	O_2	18/75	17[b]
DMSO, 0.1 equiv deferoxamine	O_2	18/75	29[b]
DMSO, 0.1 equiv EDTA	O_2	18/75	2

[a] Complete consumption of starting material.
[b] Additional, uncharacterized reaction product(s).

Veterans Affairs Medical Center (VAMC) in Providence, RI (June 2004 through September 2005). These bacteria consisted of vancomycin (glycopeptide) resistant *Enterococcus faecium* (VRE; $n = 25$), methicillin-susceptible *S. aureus* (MSSA; $n = 25$), methicillin-resistant *S. aureus* (MRSA; $n = 25$), and tetracycline-resistant *S. aureus* (TRSA; $n = 10$). The bacterial isolates were obtained from blood, sputum, tissue, and urine samples. Prior to use, all bacteria were stored in 20% glycerol and frozen at −80 °C. Drug resistance was determined for each bacterial isolate by national published guidelines that establish breakpoints based upon the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for specific antimicrobial agents.[10]

MIC and MBC values were initially measured for each compound against two clinical isolates each of MSSA, MRSA, and VRE using a broth dilution assay (Table 3). A wide range of activities was observed for both the natural products and semi-synthetic derivatives. The reduced potencies of metabolites **3** and **4** relative to **1** and **2**, respectively, demonstrated the detrimental effect of a C-1′ methoxyl substituent. VRE isolates were markedly less sensitive to the C-1′ methoxyl analogues, showing MIC values of 30- to 233-fold higher. Furthermore, the bactericidal activities were completely abolished for **3** and **4**.

Semi-synthetic modifications to the C-ring demonstrated that this portion of the molecule is vital to the antibacterial activity (Table 3). As expected, compound **5**, the dehydration product of **1**, was markedly less active than the parent compound. However, the C-4 hydroxyl analogue **6** proved to be one of the most potent compounds in this series, exhibiting MIC values of 0.11–0.22 μM against *S. aureus* and 1.8 μM versus VRE. This result indicates that retention of a hydroxyl group in the C-ring is more important than ring flexibility for maximal antibacterial activity. Installation of an aldehyde at C-12 rescued some of the activity lost by **5** against the *S. aureus* isolates, but was 4- to 15-fold less effective than **5** against VRE. Reduction of the aldehyde provided the most consistently potent agent, derivative **8**, with MIC and MBC values between 0.23 and 0.91 μM against all clinical isolates. Only analogues **1** and **8** showed potent bactericidal activity against both MSSA and MRSA, and **8** was the lone compound to possess bactericidal activity versus VRE (MBC = 0.91 μM).

Table 3. Antibacterial activities for bisanthraquinone analogues **1**–**8** against clinical isolates

Analogue	No. of isolates tested	MSSA MIC[a] range (μM)	MSSA MBC[b] range (μM)	MRSA[b] MIC range (μM)	MRSA MBC range (μM)	VRE MIC range (μM)	VRE MBC range (μM)	HCT-116[c] IC_{50} (μM)
1	2	0.05 to 0.11	0.05 to 1.8	0.11 to 0.45	0.91 to 3.6	0.24	29	2.0
2	2	0.22 to 0.88	56	0.44 to 0.88	>56	0.44 to 1.8	28	7.7
3	2	7.1 to 14	>56	7.1 to 14	>56	56	>56	1.1
4	2	0.86 to >55	>55	0.86 to >55	>55	27 to 55	>55	10.5
5	2	7.5	30 to >60	7.5	7.5	1.9 to 3.7	>60	3.4
6	2	0.11 to 0.22	3.6 to 15	0.11	29 to >58	1.8	>58	0.9
7	2	1.8 to 3.6	>58	0.23 to 0.91	>58	15 to 29	>58	3.3
8	2	0.23	0.91	0.23 to 0.91	0.91	0.23	0.91	4.0

[a] MIC, minimum inhibitory concentration.
[b] MBC, minimum bactericidal concentration.
[c] HCT-116 is a human colon tumor cell line.

Table 4. MIC_{50} values for bisanthraquinone analogues **1** and **6** against an expanded panel of clinical isolates and Gram-negative pathogens

Clinical isolates	No. of isolates tested	**1** MIC_{50} (range) in μM	**6** MIC_{50} (range) in μM
MSSA	25	0.11 (0.054–0.22)	0.45 (0.11–0.91)
MRSA	25	0.23 (0.11–0.90)	0.45 (0.23 – 0.91)
VRE	25	0.90 (0.22–3.6)	3.6 (0.91–14)
TRSA[a]	11	0.11 (0.11–0.23)	0.46 (0.11–0.91)
K. pneumoniae[b]	1	>29	>29
E. coli[c]	1	>29	>29

[a] Includes ten clinical isolates and Mu50 (ATCC 700699).
[b] *Klebsiella pneumoniae* (ATCC 700603).
[c] *Escherichia coli* (ATCC 35218).

Based on the above results and compound availability, metabolite **1** and semi-synthetic derivative **6** were selected for further evaluation against an expanded panel of pathogens. The MIC_{50} values (minimum inhibitory concentration at which 50 percent of clinical isolates are inhibited)[11] were determined by assaying against 25 clinical isolates each of MSSA, MRSA, and VRE, and ten isolates of TRSA (Table 4). Antibiotic activity was also tested against Mu50 (ATCC 700699), a TRSA that possesses a tetM ribosomal protection resistance mechanism. Overall, **1** and **6** demonstrated potent in vitro activities against all clinical VRE, MSSA, MRSA, and TRSA isolates (MIC_{50} range 0.05–1.8 μM). Both **1** and **6** were active against Mu50 with MICs of 0.23–0.46 μM, respectively. To further evaluate the spectrum of activity for these antibiotics, the compounds were evaluated against *Escherichia coli* (ATCC 35218) and *Klebsiella pneumoniae* (ATCC 35218). Neither demonstrated kill against these Gram-negative bacteria (MIC > 29 μM).

The antibacterial activities of compounds **1** and **6** were next evaluated using time-kill assays. Time-kill studies allow investigators to quantify the killing of a bacterium over a specified amount of time. This type of methodology has advantage over traditional MIC testing in that one can assess how rapidly an antibiotic kills or suppresses the growth of an isolate, and also allows for typing the antibiotic activity as bactericidal or bacteriostatic. Bactericidal activity (99.9% kill) is defined as a $\geqslant$3-$\log_{10}$ CFU/mL reduction in colony count from the initial inoculum, while bacteriostatic activity is defined as a <3-$\log_{10}$ CFU/mL reduction.[12] Inactive is defined as no observed reductions in initial inoculum.

Using a 24-h time-kill study, compound **1** demonstrated significant ($p = 0.05$) bactericidal activity (99.9%) against MSSA, MRSA, and VRE (Table 5; Fig. 2). Bisanthraquinone derivative **6** demonstrated bactericidal activity against MSSA and MRSA. There was no MIC shift increase when isolates from a 24-h time point were compared to the starting bacterium. Therefore, no

Table 5. Reduction in pathogen CFU/mL following a 24-h antibiotic exposure

Isolates ($n = 2$)	**1** Δ in 24-h $\log_{10}$ CFU/mL	**6** Δ in 24-h $\log_{10}$ CFU/mL
MSSA	−3.14 ± 0.275	−3.37 ± 0.244
MRSA	−3.17 ± 0.424	−2.98 ± 0.104
VRE	−3.00 ± 0.294	−2.59 ± 0.163

Antibiotics **1** and **6** were tested at 4 × MIC in a time-kill assay. Bactericidal activity is defined as a $\geqslant$3 $\log_{10}$ CFU/mL reduction in colony count from the initial inoculum, while bacteriostatic activity is defined as a <3-$\log_{10}$ reduction. Inactive is defined as no reduction in CFU/mL.

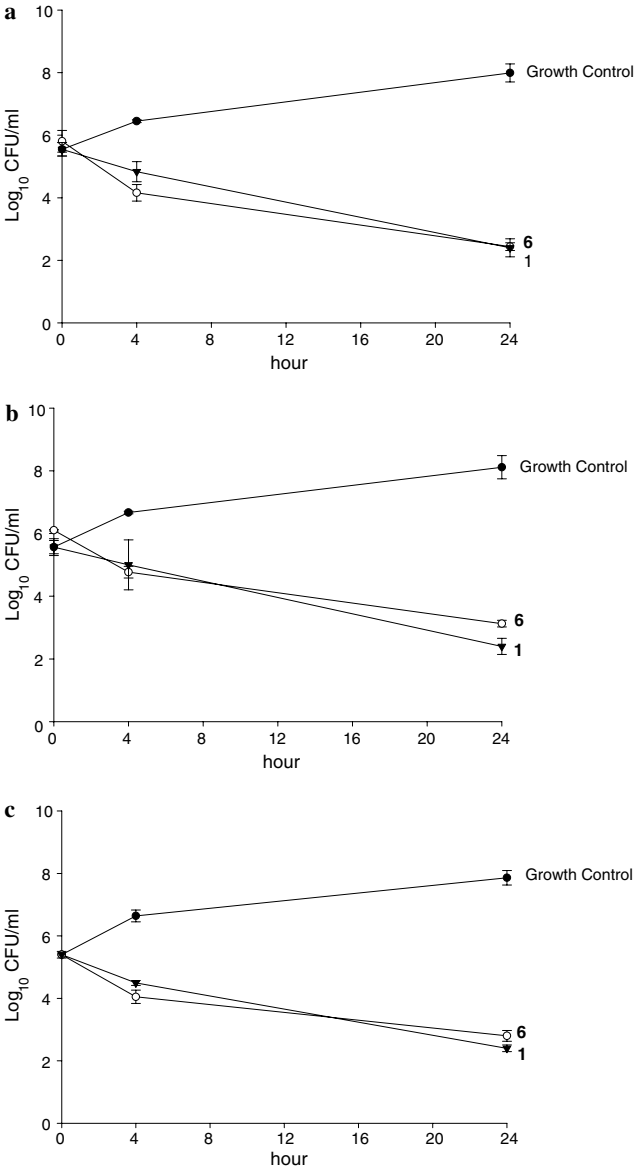


Figure 2. Time-kill assay results for antibiotics **1** and **6** at 4 × MIC against (a) MSSA, (b) MRSA, and (c) VRE.

bacterial resistance to **1** or **6** was observed during this experiment.

Each compound was further tested for cytotoxicity against the human colon tumor cell line HCT-116. All of the bisanthraquinone metabolites and semi-synthetic derivatives exhibited low micromolar activity in this assay (Table 3).

3. Discussion

New bisanthraquinone antibiotics were isolated and synthesized to further define structure–activity relationships in a potent new structural class of antibacterial agents. These rigid molecules have a fused, octacyclic skeleton that is unique among streptomycete metabolites. Modifications to the C-ring were found to have substantial effects on the antibacterial activities of these bisanthraquinones. Although conformational change imparted by an additional $\Delta^{2,3}$ C-ring double bond (analogues **5**–**8**) may slightly impact the antibacterial potency, the requirement for an oxygen bearing substituent proximal to C-3 is more vital. Submicromolar MIC values were predominately observed for bisanthraquinones **1**, **6**, and **8** across the panel of clinical pathogens. The commonality between these potent analogues is a single hydroxyl substituent attached to or neighboring C-3. The *trans* diol **2** and aldehyde **7** showed potent MICs versus MSSA, MRSA, and VRE, but their bactericidal activities (MBCs) were greatly diminished.

Evaluation of the antibiotic properties was conducted using pathogenic isolates of MSSA, MRSA, TRSA, and VRE collected from patients at the Veterans Affairs Medical Center in Providence RI. Assays conducted using clinical isolates are expected to provide a better indication of the true potential of new agents. The specific mechanisms of resistance in our clinical strains are unknown. Within TRSA isolates, clinically observed resistance is primarily due to either ribosomal protection or drug efflux.[13,14] Given structural similarities between the bisanthraquinones (A–C rings) and tetracyclines (B–D rings), we considered that these antibiotics may share a similar mechanism of action, and that tetracycline-resistant *S. aureus* may be less sensitive to these compounds. This was not the case. Compounds **1** and **6** were comparably active across the panel of TRSA, MSSA, and MRSA isolates ($MIC_{50} < 0.5$ μM). They also showed potent growth inhibition against Mu50 (ATCC 700699), a strain possessing a resistance gene that encodes for the ribosomal protection protein Tet(M) that prevents effective drug binding.[13] The bisanthraquinones presented here show consistent and potent activity across a panel of pathogens that are resistant to an array of clinically important antibiotics. However, the mechanism of action of these compounds is presently unknown.

Metabolite **1** was determined to be bactericidal against VRE, MSSA, and MRSA in time-kill studies. The semi-synthetic bisanthraquinone **6** demonstrated similar activity against MSSA and MRSA, and bacteriostatic activity against VRE. Bactericidal activity is noteworthy from a clinical perspective. When treating severe infections, a rapid clearance of a pathogen may be necessary to achieve a better prognosis, and therefore a bactericidal agent is preferred.[15,16]

The autoxidation of the allylic methyl group in **5** occurred under mild conditions. Autoxidations at allylic positions are well investigated;[17,18] however, most reported reaction conditions involve base,[19] metal,[20] electrochemical,[21] or light[22] catalysis. In the autoxidation of these bisanthraquinones, there is not a requirement for either base or light catalysis, though the reaction is accelerated in the presence of DMAP. The addition of formic acid had no effect on the reaction rate. It is curious that EDTA nearly quenched the reaction, but deferoxamine had no effect. Deferoxamine is a strong, selective iron chelating agent, whereas EDTA is known to chelate diverse metals that catalyze autoxidations (e.g., copper, iron, and cobalt). These results suggest that a trace metal other than iron may be involved in oxidative catalysis. It is also interesting that oxidation takes place at the least substituted allylic position, perhaps indicating that steric factors are controlling the regioselectivity of the reaction.[17,18] The chemical transformations observed with these conjugated compounds under mild conditions caution against the long-term storage of compounds in DMSO for bioassay testing.[23]

All of the bisanthraquinones exhibited low micromolar cytotoxicity toward HCT-116 tumor cells, and mammalian cell toxicity may be an issue with these compounds. However, tumor cell toxicity is likely not the best measure for assessing the selectivity of these compounds. Further modifications to the bisanthraquinone scaffold are warranted to improve the therapeutic index of these new antibiotics.

4. Experimental

4.1. General

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 ($CDCl_3$). Analytical HPLC was performed using a Hitachi L-7100 gradient pump with an L-7455 diode array detector and using a Waters X-Terra MS C18 5-μ (3.0 × 100 mm) column. Semi-preparative HPLC was performed using a Waters 600 pump, a 486 UV Absorbance Detector, and a Waters X-Terra Prep RP18 5-μ (19 × 100 mm) column. LC/MS experiments were conducted on an Agilent 1100 analytical HPLC coupled to an Applied Biosystems Mariner© Mass Spectrometer. 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. Solvents used for chromatography were of HPLC grade and DMSO (99.9%) used in reactions was distilled at 81 °C under 0.015 atm immediately prior to use.

4.1.1. Isolation and fermentation of marine *Streptomyces* sp. Streptomycete strain N1-78-1 (GenBank Accession No. DQ470014) was isolated from an unidentified blue green alga (URI No. N36-11-10) collected from the tunicate *E. turbinata* in La Paragua, Puerto Rico. Twenty eight 1-L cultures were shaken (150 RPM, 31 °C) for 12 days in a marine medium comprised of 4 g glycerol, 0.4 g L-asparagine and 2 g chitin per liter seawater.

4.1.2. Bioassay-guided isolation of bisanthraquinone metabolites 3 and 4. The spent culture broths were filtered through cheesecloth, extracted with ethyl acetate, and the organic layer was concentrated in vacuo to yield 506 mg of crude extract. Fractionation began with Sephadex LH20 (Fluka®) column chromatography using 3:2:1 isooctane/MeOH/toluene (2.6 × 67.5 cm column, 4 mL fractions). Fractions 30–110 were pooled (150 mg) and further purified by silica-gel (Aldrich, 60 Å, 230–400 mesh) using 40–100% EtOAc in TMP + 0.2% formic acid (2.5 × 50 cm column). Reversed-phase HPLC (10 mL/min; 40–75% MeCN in H_2O with 0.2% formic acid over 30 min) yielded 2.5 mg each of pure **3** and **4**.

4.1.2.1. Bisanthraquinone derivative 3. Yellow amorphous solid (0.1 mg/L fermentation yield); $[\alpha]_D^{22}$ +260 (*c* 0.05, MeOH), UV (MeOH) λ_{max} nm ($\log\varepsilon$) 226 (4.44), 260 (4.18), 283 sh (3.81), 364 (3.93), 398 sh (3.81); HRESIMS $[M+H]^+$ obsd *m/z* 567.1650, calcd 567.1655 for $C_{33}H_{27}O_9$ (Δ 0.9 ppm).

4.1.2.2. Bisanthraquinone derivative 4. Yellow amorphous solid (0.1 mg/L fermentation yield): $[\alpha]_D^{22}$ +350 (*c* 0.02, MeOH), UV (MeOH) λ_{max} nm ($\log\varepsilon$) 226 (4.57), 260 (4.32), 283 sh (3.94), 367 (4.06), 396 sh (3.95); HRESIMS $[M+H]^+$ obsd *m/z* 583.1605, calcd 583.1604 for $C_{33}H_{27}O_{10}$ (Δ 0.1 ppm).

4.2. Semi-synthetic derivatives

4.2.1. Bisanthraquinone derivative 5. Compound **1** (22 mg, 40 μmol) was dissolved in 25 mL of DMSO and heated at 67 °C for 24 h. The solution was passed over Amberlite XAD-7 HP resin (7 g, ACROS Organics), rinsed with distilled water (100 mL), and vacuum dried in a Buchner funnel. The products were then eluted from the resin with acetone (300 mL), concentrated in vacuo, and purified by HPLC (10 mL/min; 40–75% MeCN in H_2O with 0.2% formic acid over 30 min; t_R **1** = 25.3 min, **5** = 32.5 min). The dehydration product **5** was obtained as a yellow amorphous solid (13 mg, 61% yield). HRESIMS $[M+H]^+$ obsd *m/z* 535.1401, calcd 535.1393 for $C_{32}H_{23}O_8$ (Δ = 1.5 ppm).

4.2.2. Bisanthraquinone derivative 6. The bisanthraquinone metabolite **2** (6 mg, 10.5 μmol) was dissolved in 1.7 mL of DMSO with 0.005% H_2SO_4 and heated to 67 °C for 8 h. DMSO was removed by flash chromatography as described for **5**, and the concentrated crude product was purified by semi-preparative HPLC (10 mL/min; 40–75% MeCN in H_2O with 0.2% formic acid over 30 min; t_R **2** = 13.9 min, **6** = 19.7 min). The desired dehydration product was obtained as a yellow amorphous solid (5 mg, 87% yield). HRESIMS $[M+H]^+$ obsd *m/z* 551.1341; calcd 551.1342 for $C_{32}H_{23}O_9$ (Δ 0.1 ppm); UV (MeOH) λ_{max} nm ($\log\varepsilon$) 226 (4.62), 257 (4.37), 287 (4.02), 401 (4.19). IR (neat) ν_{max} 3400 (br), 3023, 2917, 2843, 1739, 1599, 1492, 1451, 1374, 1265, 754 cm^{-1}; ^{13}C NMR (100 MHz, $CDCl_3$) δ 189.4 (C-9′), 187.0 (C-9), 181.9 (C-10′), 169.6 (C-1), 162.6 (C-1′), 161.6 (C-8), 157.2 (C-8′), 153.3 (C-3), 148.3 (C-3′), 138 (C-7′), 137.5 (C-6), 136 (C-10a), 135 (C-10a′), 132.6 (C-4a′), 129.3 (C-6′), 124.3 (C-2′), 122.3 (C-2), 121.5 (C-5′), 120.6 (C-4′), 119.1 (C-5, C-10), 118.2 (C-7), 116.6 (C-8a′), 114.3 (C-9a′), 113.5 (C-8a), 97.0 (C-9a), 86.3 (C-11), 83.2 (C-4), 59.6 (C-4a), 22.2 (C-14), 19.6 (C-13), 14.0 (C-12).

4.2.3. Bisanthraquinone derivative 7. A solution of **5** (0.2 mg, 0.37 μmol) in 1 mL of DMSO was heated at 75 °C under O_2. The O_2 atmosphere was replenished every 24 h by sparging the reaction for two minutes. LC/MS analysis with additional UV detection (λ = 254 nm) showed the reaction was complete after 124 h (1 mL/min; 40–75% MeCN in H_2O + 0.2% formic acid over 30 min; t_R **5** = 24 min, **7** = 22.7 min). The desired product was purified by HPLC (10 mL/min; 40–75% MeCN in H_2O with 0.2% formic acid over 30 min) to provide the pure aldehyde as a yellow/orange amorphous solid (0.13 mg, 66% yield). HRESIMS $[M+H]^+$ obsd *m/z* 549.1188; calcd 549.1186 for $C_{32}H_{21}O_9$ (Δ 0.5 ppm); UV (MeOH) λ_{max} nm ($\log\varepsilon$) 201 (4.91), 220 (4.91), 235 (4.41), 258 (4.46) 405 (4.32). IR (neat) ν_{max} 3550, 3064, 1634, 1585, 1454, 1348, 1241, 1176, 845, 771 cm^{-1}; ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.5 (C-9′), 190.6 (C-12), 186.7 (C-9), 181.9 (C-10′), 166.1 (C-1), 162.7 (C-1′), 162.3 (C-8), 155.3 (C-8′), 148.2 (C-3′), 143 (C-3), 139 (C-7′), 138.5 (C-6), 138 (C-2), 137.9 (C-10a), 135.2 (C-10a′), 132.6 (C-4a′), 127.8 (C-6′), 124.4 (C-2′), 121.7 (C-5′), 120.6 (C-4′), 120.4 (C-10), 119.4 (C-7), 118.8 (C-5), 118 (C-8a′), 114.4 (C-9a′), 113.9 (C-8a), 100.7 (C-9a), 85.7 (C-11), 54.9 (C-4a), 49.5 (C-4), 22.2 (C-14), 20.4 (C-13).

4.2.4. Bisanthraquinone derivative 8. Aldehyde **6** (2.9 mg, 5.3 μmol) was treated with 500 μL of a 10.6 mM solution of $NaBH_4$ in anhydrous THF solution and stirred. The reaction was monitored by normal phase TLC (3% MeOH in DCM + 0.1 formic acid; R_f **7** = 0.9, **8** = 0.3). After 15 min, the reaction was quenched with MeOH and subjected to semi-preparative HPLC purification (10 mL/min; 0–100% MeCN in H_2O with 0.2% formic acid over 60 min; t_R **7** = 23 min, **8** = 31 min). The desired product was obtained as a yellow amorphous solid (1.4 mg, 48% yield). HRESIMS $[M+H]^+$ obsd *m/z* 551.1356, calcd for $C_{32}H_{23}O_9$ 551.1342 (Δ 2.6 ppm).

4.3. Biological activity and in vitro assay

4.3.1. Susceptibility testing. Minimum inhibitory concentrations (MIC) and resistance testing were determined in duplicate using methods described in documents published by The Clinical and Laboratory Standards Insti-

tute (formerly, National Committee for Clinical Laboratory Standards).[10] Fresh cation-adjusted Mueller–Hinton broth (SMHB; Difco Laboratories, Sparks, MD, USA; 25 μg/mL calcium and 12.5 μg/mL magnesium) was used to dilute antimicrobial agents in a serial 2-fold schedule. Colony counts for the inoculum verification was determined using Tryptic Soy Agar plates (TSA, Difco, Becton Dickinson Co., Sparks, MD, USA). MIC was defined as the lowest concentration of an antimicrobial agent visually inhibiting more than 99% of the colonies. Following incubation, 100 μL of broth was subcultured onto TSA for MBC determination. The MBC was defined as the lowest antibiotic concentration to show no growth (99.9% kill) after 24 h of incubation. MIC and MBC determination can be found in Table 3. Control isolates were obtained from the American Type Culture Collection (ATCC) and were as follows: MSSA (ATCC 25923) and MRSA (ATCC 43300), *K. pneumoniae* (ATCC 700603), and *E. coli* (ATCC 35218). There was no control strain for VRE.

4.3.2. In vitro time-kill assay. For time-kill experiments, fresh SMHB was used to determine the antibiotic activity against randomly selected VRE ($n = 2$), MSSA ($n = 2$), and MRSA ($n = 2$). Each time-kill was performed as previously described in duplicate and bacterial quantification of all samples were performed on TSA.[24] All antimicrobial agents were tested at four times their respective MIC with a starting inoculum of 5×10^5 CFU/mL. Three to five colonies from overnight growth on TSA at 37 °C were added to normal saline and adjusted to produce a 0.5 McFarland standard. This suspension was then diluted with SMHB to achieve an inoculum of 1×10^6 CFU/mL. A 0.2 mL suspension of each organism was added to 1.8 mL of SMHB with a 0.1 mL stock solution of each antibiotic in a 24-well tissue culture plate (final volume, 2.0 mL per well). Culture wells were incubated at 35 °C with constant shaking for 24 h. Sample aliquots (0.1 mL) were removed from cultures at 0, 4, 8, and 24 h. Antimicrobial carryover was minimized by serial dilution (10- to 10,000-fold) of plated samples. Colony counts were determined by plating 20 μL of each diluted sample onto TSA plates in triplicate, followed by incubation at 35 °C for 24 h. This methodology has a lower limit of detection of 2.0 $\log_{10}$ CFU/mL. Growth control wells for each organism were prepared without antibiotic and run in parallel to the antibiotic test wells.

4.3.3. Activity defined. Bactericidal activity (99.9% kill) is defined as a $\geqslant 3$ $\log_{10}$ CFU/mL reduction in bacterial density from the starting inoculum.[12,25] Time to 99.9% kill was determined by visual inspection.

4.3.4. MIC increase. Development of a MIC increase was evaluated at the 24 h time point (time kill) for each of the four randomly selected isolates. One hundred microliters samples from the 24 h time points were plated on TSA containing 4-fold the MIC of the respective antibiotic to assess the development of resistance. Plates were examined for growth after 24 and 48 h of incubation at 35 °C. No MIC shifts were observed.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2006.08.038.

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