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Arenimycin, an antibiotic effective against rifampin- and methicillin-resistant *Staphylococcus aureus* from the marine actinomycete *Salinispora arenicola*

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Keywords

Salinispora arenicola; antibiotic; rifampin- and methicillin-resistant *Staphylococcus aureus*

The incidence of infections by drug-resistant bacteria continues to increase and remains a serious threat to human health. In 2007, the US CDC (Centers for Disease Control and Prevention) reported that the number of serious infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) alone was close to 100 000 a year, with almost 19 000 related fatalities, a number that is larger than the US death toll attributed to HIV/AIDS (human immunodeficiency virus/acquired immunodeficiency syndrome) in the same year.1 Marine natural products offer a rich source of poorly explored bioactive molecules that are of potential value in the development of new pharmaceutical agents.2 Marine bacteria in particular have proven to be a good source of small molecules, with the vast majority of compounds being discovered from chemically prolific Gram+ bacteria belonging to the order Actinomycetales (actinomycetes). Of the marine actinomycetes from which novel natural products have been reported, the obligate marine genus *Salinispora* has yielded an impressive array of structurally diverse compounds including salinosporamide A, which is currently being used in clinical trials for the treatment of cancer.[3] These compounds are produced in species-specific patterns and include potent antibiotics in the rifamycin class, which are consistently observed in extracts of *Salinispora arenicola*.[4] In an effort to discover new antibiotics from a large collection of *S. arenicola* strains, a rifampin-resistant strain of MRSA was used to screen extracts for antibiotic activities that are derived from compounds other than rifamycins.

In total, we screened ca. 2400 crude extracts from a library that was heavily enriched with extracts collected from *Salinispora* species. Only six (0.3%) of the extracts showed promising biological activity against rifampin-resistant MRSA. Among these active extracts, a liquid chromatography (LC) MS analysis of the extract of *S. arenicola* strain CNR-647 showed peaks of several metabolites, some of which were identified by searching our LCMS-UV-Vis database to be known staurosporine and rifamycin analogs. However, one unknown metabolite

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was observed, which by its UV-Vis absorption characteristics, was observed to likely be a quinone derivative. A 5 l culture broth of this organism yielded 450 mg of crude extract, which on bioassay-guided fractionation, followed by purification of the active fraction, led to the isolation of a new antibiotic, arenimycin (**1**, Figure 1). Arenimycin belongs to the benzo[α] naphthacene quinone class of antibiotics and is most closely related to SF2446B1 (**2**) produced by *Streptomyces* sp. SF2446, which possesses a methoxyl rather than a hydroxyl substituent on the 4′ -position of the sugar.[5] This new structural derivative is the first report of this class of antibiotics from the marine actinomycete *S. arenicola*. Herein, we report the isolation, structure elucidation and potent antibiotic activity of arenimycin against a panel of drug-resistant human pathogens.

The *S. arenicola* strain CNR-647 was isolated from a sample of the ascidian *Ecteinascidia turbinata* collected from a mangrove channel at Sweetings Cay, Grand Bahama Island (tropical Atlantic Ocean) in June 2002. The animal was washed with sterile seawater, ground with an alcohol-sterilized mortar and pestle. A volume of 1 ml of the resulting suspension was added to 4ml of sterile seawater, heated at 55 °C for 6 min, and 50 μl of the resulting suspension was plated onto seawater agar containing 5μg ml^{-1} rifamycin and 100μg ml^{-1} cycloheximide. The strain was identified as *S. arenicola* on the basis of the 16S rRNA sequence analysis (GenBank accession number FJ887039).

The CNR-647 strain was cultured in 5×1l volumes at 27°C for 7 days while shaking at 215 r.p.m. in the nutrient medium A1Bfe, composed of 10 g starch, 4 g yeast extract, 2 g peptone, 40mg $Fe_2(SO_4)_3$ $4H_2O$, 100mg KBr, per 1 liter filtered natural seawater. Amberlite XAD-7 resin (20 gl^{-1}) was added at the end of the fermentation period to adsorb the secreted secondary metabolites. The culture and resin were shaken at 215 r.p.m. for two additional hours. The resin and cell mass were collected by filtration through cheesecloth, and the residue was washed with DI water to remove salts. The resin, cell mass and cheesecloth were then soaked for 2 h in acetone. Next, the acetone extract was filtered and solvent removed under vacuum to yield 0.45 g of crude extract from a 5 l culture.

The crude extract was fractionated by bioassay-guided methods using reversed-phase C18 vacuum LC (H_2O/CH_3OH; gradient 90:10 to 0:100%) to yield 8 fractions. The active fraction 4 was then fractionated by preparative RP HPLC (Prep Nova-Pak HR C18, 6 μm, 300 ×40 mm^2) with CH_3CN/H_2O as eluent, followed by semi-preparative isocratic HPLC to yield pure arenimycin (**1**) as a noncrystalline red solid (3.5 mg, Table 1).

The molecular weight of arenimycin (**1**) was obtained from the analysis of high-resolution electrospray ionization time-of-flight mass spectral data, which showed pseudomolecular ions at *m/z* 668.2035 $[M+H]^+$ and 690.1753 $[M+Na]^+$. These data established the molecular formula of **1** as $C_{33}H_{33}O_{14}N$, which indicated the presence of 18 double-bond equivalents. The ^{13}C NMR spectrum of **1** showed 33 signals, consistent with the molecular formula. The UV spectrum showed absorption maxima at 216, 228, 251, 300, 415 and 480nm (CH_3OH), which indicated the presence of a complex naphthoquinone moiety (Table 2). ^{13}C and gHSQC NMR experiments showed the presence of two methyl carbons, two methylene carbons, five methine carbons, three methoxyl carbons, two quaternary carbons, three sp^2 methine carbons, eleven sp^2 quaternary carbons and five carbonyl carbons. The ^1H-^1H COSY and gHSQC NMR spectral data allowed two spin systems to be observed, an *N*-glycosyl sugar, and an ethylene unit. Detailed analysis of gHSQC, and gHMBC data from **1** showed two partial structures, I and II, as shown in Figure 2. The *N*-glycosyl sugar moiety of partial structure I was assigned by the analysis of combined one-dimensional (1D) and two-dimensional (2D) NMR data. The ^1H NMR spectrum of compound **1** showed an anomeric proton signal at δ 4.69 (^1H, d, J=9.0 Hz) and four −CH–OH signals between δ 3.67 and δ 3.34, which defined a hexopyranosyl ring. However, the carbon chemical shift for the anomeric carbon was δ 79.4 p.p.m., suggesting

an *N*-substitution rather than the common *O*-substitution. On the basis of 1D and 2D NMR data, the sugar moiety was identified as 2′-*O*-methyl-rhamnopyranose.

In a similar manner, a comprehensive analysis of 2D NMR data (Table 2) allowed the remaining two rings of the anthraquinone substructure II to be assigned. Finally, the CH proton signal at δ 8.20 from substructure I, showed key HMBC correlations with the quinone carbonyl, C-14, of substructure II, and the hydroxyl at C-8 showed a weak correlation with C-7, thus allowing these two substructures to be connected to provide structure **1**.

An AntiBase[5] search with the above-mentioned carbon skeleton showed that this compound was similar to SF2446B1 (**2**),[6] the difference between the two compounds being in the hexopyranose sugar of arenimycin (**1**). A literature search showed that the skeleton of quinones with this ring system are very rare in nature, only observed in collinone,[7] ansacarbomitocins,[8] G-2N, G-2A,[9] KS-619[10] and BE-19412A.[11]

The overall stereostructure of **1** was assigned to be identical to that of SF2446B1. Comparison of the ^{13}C NMR data for these two metabolites showed only very minor variations in the order of ≤ 0.3 p.p.m. Given the high degree of analogy, arenimycin can be assumed to have the identical stereostructure at the ring juncture as SF2446B1 (**2**). We attempted to record the optical rotation ($[\alpha]_D$) of **1**, but were unable to obtain confident results because of the intense red color of this compound. The $[\alpha]_D$ for the related compound **2** was also not reported, presumably for the same reason. The relative configuration of the anomeric carbon in the pyranohexose moiety in arenimycin (**1**) was assigned as β, on the basis of an observed NOE among H-1′, H-3′ and H-5′ and also from the analysis of vicinal proton coupling constants. NOE data also confirmed the relative configurations of the substituents at C-2′, C-3′, C-4′ and C-5′.

The antibiotic activities of arenimycin were evaluated using various drug-resistant *Staphylococcus* and *Enterococcus* strains, as well as the human colon adenocarcinoma cell line HCT-116. Human pathogenic bacterial strains were obtained from the Clinical Microbiology Laboratory at the San Diego VA Healthcare System. Antibacterial testing for the majority of the drug-resistant bacteria was conducted using the broth microdilution method in 96-well plates. Antibacterial activity against *Mycobacterium bacille* was performed using the agar dilution method with Middlebrook 7H10 agar.

In the HCT-116 adenocarcinoma cytotoxicity screen, compound **1** was a potent cell-division inhibitor showing IC_{50}=1.16μg ml^{-1}. Antibacterial testing with a panel of human Gram+ pathogens, such as various MRSA strains, *Enterococcus feacalis* and *Enterococcus faecium*, showed that arenimycin (**1**) exhibited MIC values at or < 1 μg ml^{-1} (Table 3). Arenimycin was not active against one vancomycin-resistant *Enterococcus* isolate (Table 3), but inhibited the growth of *M. bacille* Calmette Guerin at 1 μg ml^{-1}.

In summary, we show herein that using a rifampin- and methicillin-resistant *S. aureus* strain has resulted in the discovery of a new antibiotic from strains that produce significant amounts of antibiotic rifamycin derivatives. Arenimycin (**1**) exhibits potent antimicrobial activities against drug-resistant *Staphylococci* and some other Gram-positive organisms and one *Mycobacterium* strain. The fact that arenimycin also inhibits eukaryotic cell division likely illustrates a nonselective mechanism of cytotoxic action, and may explain why this class of antibacterial agents has not been further explored.

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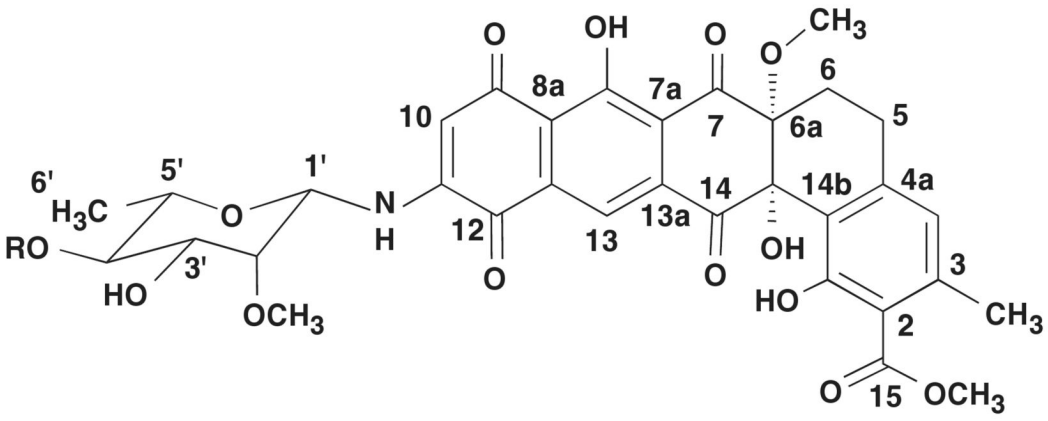

Figure 1.
Structures of arenimycin (**1**, R=H) and SF2446B1 (**2**, R=CH_3).

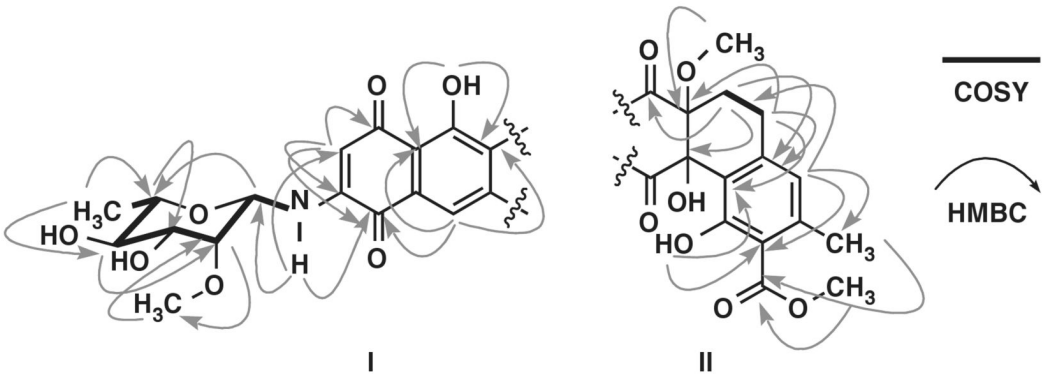

Figure 2.
Key ^{1}H-^{1}H COSY and HMBC correlations observed in substructures I and II for arenimycin (**1**).

Table 1

Physicochemical properties of 1

Appearance[a]	**Dark red solid, mp=207 °C**
HR-ESI-MS (*m/z*)	$[M+H]^+$=668.2035 for $C_{33}H_{33}O_{14}N$+H
Found:[b]	$[M+Na]^+$=690.1753 for $C_{33}H_{33}O_{14}N$+Na
UV (CH_3OH) λ_{max}	216 (4.23), 228 (4.02), 251 (3.85), 300 (2.80), 415 (2.18),
(log ε)nm[c]	480 (2.16).
IR (thin film) ν_{max}	3450, 3380, 2930, 1725, 1675, 1650, 1620, 1560, 1509,
cm^{-1}[d]	1364, 1310, 1260, 1160, 1110, 950, 801.

[a] Melting points were determined on a Mel-Temp apparatus and are uncorrected.

[b] HRMS were recorded on ThermoFinnigan MAT900XL (Thermo Scientific, Waltham, MA, USA) with an Agilent ESI-TOF (Agilent, Santa Clara, CA, USA) at The Scripps Research Institute, La Jolla.

[c] UV spectra were recorded on a Perkin-Elmer Lambda 19 UV/vis spectrophotometer (Perkin-Elmer, Waltham, MA, USA) with a path length of 1cm.

[d] IR spectra were acquired as a thin film (NaCl) on a Perkin-Elmer 1600 series FTIR spectrometer.

Table 2

NMR spectral data for arenimycin (1) in $CDCl_3$

No. C/H	δ_C	δ_H (J in Hz)	COSY	HMBC	NOESY
1	160.3 C				
2	109.6 C				
3	142.8 C				
4	124.1 CH	6.49, s	H-16 (*w*)	2, 5, 14b, 15, 16	H-5β, H-16
4a	145.0 C				
5β	26.8 CH_2	3.08, ddd (19.2, 8.6, 2.0)	H-6β, H-6α	4a, 6, 6a, 14b	H-4
5α		3.15, ddd (19.2, 9.8, 7.2)	H-6β, H-6α	4a, 5, 14b	
6β	18.9 CH_2	2.24, ddd (12.3, 9.8, 8.6)	H-5β, H-5α	5, 6a, 7	
6α		2.75, ddd (12.3, 7.2, 2.0)	H-5β, H-5α	4a, 5, 6a, 14a	
6a	87.4 C				
7	190.1 C				
7a	124.0 C				
8	162.8 C				
8a	118.8 C				
9	189.0 C				
10	104.6 CH	5.86, s		8, 8a, 9, 11, 12	H-1′
11	147.4 C				
12	179.1 C				
12a	136.5 C				
13	116.2 CH	8.20, s		7a, 8a, 12, 14	
13a	140.9 C				
14	198.1 C				
14a	78.0 C				
14b	121.2 C				
15	172.4 C				
16	23.9 CH_3	2.35, s		2, 3, 4, 15	H-4
15OCH_3	51.9 CH_3	3.81, s		15	
6aOCH_3	52.2 CH_3	3.22, s		6a	

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No. C/H	δ_C	δ_H (J in Hz)	COSY	HMBC	NOESY
1′	79.4 CH	4.69, d (9.0)	NH-11, H-2′	2′	H-10, H-2′, H-5′
2′	80.2 CH	3.67, m	H-1′, H-3′	3′, 2′-OCH_3	H-1′, H-3′
3′	72.1 CH	3.46, m	H-2′, H-4′	1′, 4′, 5′	H-2′, H-5′
4′	75.4 CH	3.65, m	H-3′, H-5′	5′	H-6′
5′	73.7 CH	3.34, dq (9.2, 5.9)	H-4′, H-6′	3′	H-1′, H-3′
6′	18.4 CH_3	1.34, d (5.9)	H-5′	5′, 4′	H-4′
2′OCH_3	62.8 CH_3	3.75, s		2′	
1-OH		12.0, s		1, 2, 3, 14b	
8-OH		14.1, s		7a, 8, 8a	
11-NH		6.82, d (9.0)	H-1′	10, 12, 1′	

Abbreviation: *w*, weak correlation observed.

^1H, ^{13}C and two-dimensional (2D) NMR spectral data were obtained on Varian Inova 500 MHz and Varian Inova 300 MHz NMR spectrometers (Varian, Palo Alto, CA, USA). The solvent peaks at δ_C 77.0 and δ_H 7.26 were used as chemical shift internal standards.

Table 3
Gram-positive antimicrobial activity for arenimycin (1)

Organism (strain)	MIC μg ml^{-1}
Rifampin and methicillin-resistant SA	1.06
MRSA (5158)	0.53
MRSA (5085)	1.03
MRSA (5167)	0.13
MRSA (5177)	0.05
MRSA (5218)	1.00
Coagulase-negative *Staphylococcus* (52444)	0.05
Coagulase-negative *Staphylococcus* (5187)	0.05
Staphylococcus saprophyticus	0.10
Enterococcus faecalis (6653-3)	0.06
Enterococcus faecium (4733)	0.25
VR *E. faecium*	>8
Mycobacterium bacille (Calmette Guerin)	1.0

Abbreviation: MRSA, methicillin-resistant *Staphylococcus aureus*.