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Structure–activity studies of echinomycin antibiotics against drug-resistant and biofilm-forming *Staphylococcus aureus* and *Enterococcus faecalis*

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ABSTRACT

Four echinomycin antibiotics were isolated from the culture broth of a marine streptomycete, and their structures were determined by a combination of chemical and spectroscopic analyses. Antibiotic activities were measured against drug-resistant and biofilm-forming strains of *Staphylococcus aureus* and *Enterococcus faecalis*. Minimum inhibitory concentrations ranging from 0.01 μM to greater than 14 μM clearly defined structure–activity relationships for antibiotic potency. Echinomycin was the most active compound with a MIC of 0.03 μM against methicillin-resistant *S. aureus* and 0.01 μM against biofilm-forming *E. faecalis*.

Nosocomial infections caused by antibiotic-resistant staphylococci and enterococci present increasing treatment challenges, often resulting in prolonged hospitalization.[1] Biofilm growth of methicillin-resistant *Staphylococcus aureus* (MRSA) is common during hemodialysis[2] and among patients bearing indwelling medical devices.[3,4] In the United States, 12–25% of MRSA catheter-related blood stream infections result in mortality, with annual patient care costs projected to be as high as $460 million.[5] When embedded within a biofilm, MRSA demonstrates enhanced resistance to multiple classes of antibiotics.[6] Clinical isolates of enterococci are also well documented to form biofilms;[7] however, no currently available chemotherapeutic agent can completely eradicate biofilms formed by vancomycin-resistant *Enterococcus* (VRE).[8] Clearly there is a critical need for new antibiotics to combat these evolving pathogens.

Echinomycin is an antibiotic produced by *Streptomyces* bacteria,[9] and the first known bifunctional DNA bisintercalator.[10,11] Resurgent interest in its antibacterial properties derives from proven efficacy against antibiotic-resistant pathogens.[12,13] For example, echinomycin exhibited seven times the potency of vancomycin when treating systemic MRSA infections in mice.[13]

In this study, an echinomycin-producing bacterium was isolated from a marine sediment sample collected from a depth of 13.5 m in Fisher's Island Sound, New York (41°15′56″N, 72°2′30″W). Strain URI-F39 was cultivated on marine agar and identified as a *Streptomyces* sp. using 16S rRNA gene sequence comparison (Genbank Accession No. EU998645).[14] The organic extracts of 1 L fermentations showed antimicrobial activity against *S. aureus* (ATCC 43300). The broths from scale-up fermentations (16× 1200 mL) were filtered to remove cells, extracted with HP-20 resin (Diaion®, 5 × 36 cm resin bed) and eluted with 1:1, MeOH:acetone. The cells were lyopholized and extracted with 1:1, MeOH:DCM. The combined extracts were concentrated to dryness, and then partitioned between 15% aqueous MeOH and isooctane. MeOH was removed from the polar fraction in vacuo, and the remaining water was extracted sequentially with EtOAc and 9:1 DCM:isopropanol. The organic fractions were concentrated to dryness (2.75 g) and separated by LH-20 (Fluka®) column chromatography (100% MeOH, 2.6 × 67.5 cm resin bed). Early eluting fractions were combined by LC–MS analysis and subsequently purified by reversed-phase HPLC (Phenomenex Luna Phenyl Hexyl column, 5 mm, 250 × 10 mm). A linear gradient of 50–100% MeCN in H_2O + 0.1% TFA (45 min, 5 mL/min) yielded pure compounds **1** (22.6 mg), **2** (5.5 mg), **3** (16.4 mg), and **4** (7.3 mg).

The molecular formula of **1** ($C_{51}H_{65}N_{12}O_{12}S_2^+$) was established by HR-ESI-MS, and ^1H and ^{13}C NMR spectra resembled those reported for echinomycin.[15,16] Since chemical shift assignments that distinguish the upper and lower hemispheres were not previously reported, the compound was completely characterized using

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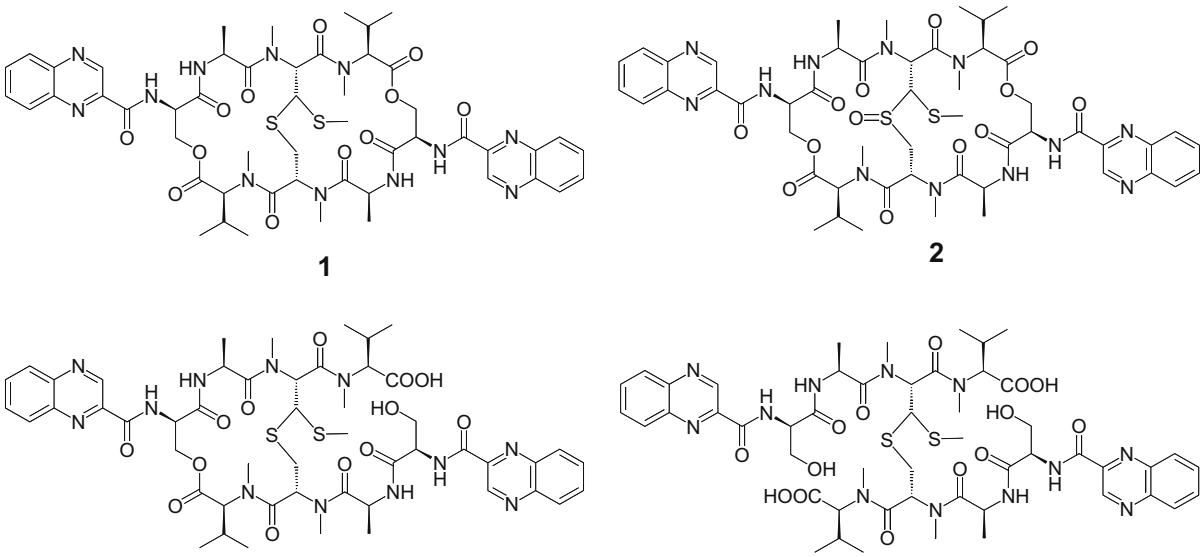

NMR spectral data. By referencing the cysteine β-protons of the thioacetal bridge, 2D NMR experiments allowed assignment of all resonances (Tables 1 and 2 in Supplemental data).

HR-ESI-MS measurements of **2** indicated a molecular formula ($C_{51}H_{65}N_{12}O_{13}S_2^+$), which required the addition of an oxygen atom to **1**. A new IR band was observed at 1014 cm^{-1}, consistent with the presence of a sulfoxide. Cys and Cys′ sulfoxide analogs of **1** have been reported in the semi-synthetic and natural product literature, respectively.[17,18] To determine the site of oxidation in **2**, ESI-MS–MS experiments were conducted on both **1** and **2** under identical fragmentation parameters. Product ions showing a loss of SCH_3 were observed for both parent compounds (*m/z* for **2** = 1068.4 $[M-H]^-$), thus indicating that oxidation resided at the Cys′ sulfur atom. The structure of **2** was confirmed by 2D NMR (Fig. 1) and shown to match that of FD-991, a natural product.[17]

The new compound depsiechinoserine (**3**) displayed a HR-ESI-MS of *m/z* 1119.4384, consistent with the addition of H_2O to the molecular formula of **1**. The UV spectrum was nearly identical to the aforementioned peptides, and selective ion monitoring LC–MS showed a very slight separation of **3** into a pair of isomers in an approximate 1:1 ratio. However, no conditions were found that allowed sufficient resolution for individual analysis. Therefore, all NMR spectra were recorded on the mixture of positional isomers, which precluded some NMR chemical shift assignments. Despite signal overlap, the spectra of **3** were highly similar to the other echinomycin peptides. A DEPT-135 experiment distinguished the quinoxaline chromophores by their characteristic ^{13}C resonances. COSY, HSQC, and HMBC allowed for the identification of two *N*-Me-Val, two Ala, and two modified Cys residues. The HSQC spectrum of **3** also identified a pair of upfield serine β-protons ($\delta_{H,H}$ 3.99, 4.09) that were distinguishable from its ester-linked serine β-protons ($\delta_{H,H}$ 4.89, 4.44). Semi-synthetic preparation of both isomers was achieved by mild hydrolysis of **1**. A solution of **1** (0.114 μmol in 50 μL THF) was treated with 125 μL of aqueous LiOH (65 mM) for 10 min at ambient temperature. The reaction was quenched with 25 μL of 0.1 N HCl, and analyzed by LC–MS–MS (5-90% MeCN in H_2O + 0.2% acetic acid, 500 μL/min over 20 min, Waters X Terra C18 MS column, 5 μm, 3 × 100 mm). Both the semi-synthetic derivatives and the natural products displayed matching retention times (18.6 min/18.6 min), and produced several identical mass fragments. Product ions in each MS^2 spectra showed loss of SCH_3 (1071.4), *N*-Me-Val (988.3), Ala′ (805.3), and Cys′ (444.1) from the parent ions (Fig. 2). Combined, these results demonstrate that **3** is the *N*-Me-Val carboxylic acid analog of echinomycin.

Compound **4** provided HR-ESI-MS data that matched the metabolite echinoserine,[19] the bis-*N*-Me-Val carboxylic acid analog of **1**. Tandem MS experiments displayed the same fragment ions as its reported structure. Since complete chemical shift assignments for this compound were missing from the literature, a full charac-

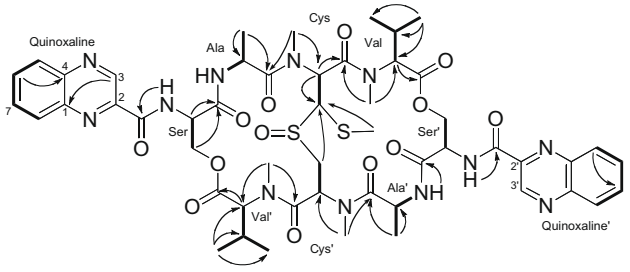

Figure 1. Selected TOCSY and COSY (bold) and HMBC correlations (arrows) observed for **2**.

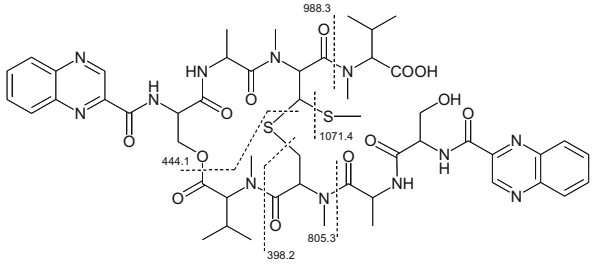

Figure 2. MS^2 fragments of depsiechinoserine (**3**) observed for both the isolated metabolite and the identical compound produced by semi-synthesis.

Table 1
Pharmacological activities for echinomycin analogs

Analog	MRSA-L32[a]		MRSA-L44[b]		MSSA[c]		EF[d]		HCT-116[e]
	MIC (μM)	MBC (μM)	MIC (μM)	MBC (μM)	MIC (μM)	MBC (μM)	MIC (μM)	MBC (μM)	IC_{50} (μM)
1	0.03	0.91	0.03	0.45	0.01	0.45	0.01	0.11	<0.003
2	1.79	7.17	1.79	7.17	0.90	3.58	0.22	>14	0.4
3	>14	>14	>14	>14	>14	>14	14	>14	4.6
4	>14	>14	>14	>14	>14	>14	>14	>14	13.4

[a] MRSA-L32 = clinical isolate.
[b] MRSA-L44, clinical isolate.
[c] MSSA, ATCC strain 35556.
[d] EF, ATCC strain 29212.
[e] HCT-116, human colon tumor cell line.[26]

terization was accomplished by examination of 2D NMR data including COSY, TOCSY, HSQC and HMBC (Tables 1 and 2 in Supplemental data). As seen with **3**, the serine β-protons of **4** were shifted upfield ($\delta_{H,H}$ 4.00, 4.04), and integration of four protons indicated that both esters were hydrolyzed in the latter compound. Additionally, the ^{13}C chemical shifts of the *N*-Me-Val and *N*-Me-Val′ carboxylic acids were shifted approximately 4 ppm downfield (δ 174.2, 174.7) as compared with **1** and **2**.

Application of the advanced Marfey's analysis established the presence of L-Ala, D-Ser, and L-*N*-Me-Val in **1**–**4** by comparison with the appropriate amino acid standards.[20] Approximately 0.5 mg of each compound was hydrolyzed with 0.8 mL of 6 N HCl in sealed glass ampoules (22 h, 95 °C). The 2,4-dinitrophenyl-5-L-leucinamide derivatives were prepared according to previously reported methods.[21] Chromatography was performed on a Waters X-Terra C18 MS column (5 μm, 3 × 100 mm) using a linear gradient (10–60% MeCN in H_2O + 0.2% acetic acid, 500 mL/min over 18 min, see Supplemental data for RT comparison). The optical rotation of **1** matched the reported value[10] and the CD spectra of all compounds displayed positive Cotton effects at approximately 205 and 280 nm and negative effects at approximately 230 and 315 nm, respectively. Therefore, the absolute configurations of **2**–**4** were determined to be the same as echinomycin.[22]

Minimum inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) were measured against biofilm-forming (BF) strains of methicillin-susceptible *S. aureus* (MSSA, ATCC 35556) and *E. faecalis* (EF, ATCC 29212).[23,24] Additionally, two clinical BF strains of MRSA (L32 and L44) were tested.[25] The clinical strains were obtained from blood samples of patients at the Providence Veterans Affairs Medical Center. Assays conducted using clinical isolates may provide a better indication of the antibiotic potential of a new agent. Each compound was also tested for cytotoxicity against a human colon tumor (HCT-116) cell line (Table 1).

Based on these results, time-kill assays were conducted on **1** and **2** allowing measurement of their cell killing properties over 24 h. The advantage of this technique over traditional MIC and MBC assays is that one can quantify how rapidly killing occurs and then determine if a compound has bactericidal or bacteriostatic activity. The time-kill studies were conducted at two and four times the respective MIC (Table 2).[27] FD-991 (**2**) demonstrated bactericidal activity against the biofilm-producing MSSA (ATCC 35556) with a decrease of 3.39 and 3.24 $\log_{10}$ CFU/mL at two and four times the MIC, respectively. Both echinomycin (**1**) and FD-991 (**2**) demonstrated bacteriostatic activity against the clinical MRSA strains and *Enterococcus faecium* at four times the MIC.

The biofilm inhibitory effects of **1** and **2** were measured using a colorimetric microtiter plate assay.[25] Biofilm formation was quantified by staining with crystal violet and measuring UV absorbance at 570 nm. Compounds **1** and **2** were evaluated over a concentration range of 0–14 μM. Each bacterial strain produced a robust biofilm in the positive controls, and all test isolates provided an acceptable optical density (OD_{570}) between 0.5 and 0.8 AU. Negative controls wells contained media only. Echinomycin (**1**) and FD-991 (**2**) inhibited all *S. aureus* and *E. faecium* isolates at concentrations near their MIC (Fig. 3).

Table 2
Time-kill assay results for **1** and **2** against *S. aureus* and *E. faecalis* strains

Test	Mean change in bacterial density[a] ($\log_{10}$ CFU/mL)			
	MRSA-L32	MRSA-L44	MSSA	EF
Growth control	3.44 ± 0.22	3.21 ± 0.05	2.84 ± 0.03	2.87 ± 0.09
2× MIC				
1	0.61 ± 0.23	0.423 ± 0.09	−1.54 ± 0.06	0.04 ± 0.09
2	−1.35 ± 0.20	−1.09 ± 0.19	−3.39 ± 0.06	0.69 ± 0.12
4× MIC				
1	−2.39 ± 0.09	−1.23 ± 0.03	−1.79 ± 0.04	−1.42 ± 0.11
2	−2.15 ± 0.17	−2.24 ± 0.07	−3.24 ± 0.17	−1.04 ± 0.09

[a] Inoculum change from starting inoculum of 5 × 10^5 CFU/mL (observed at 24 h).
CFU, colony forming units.

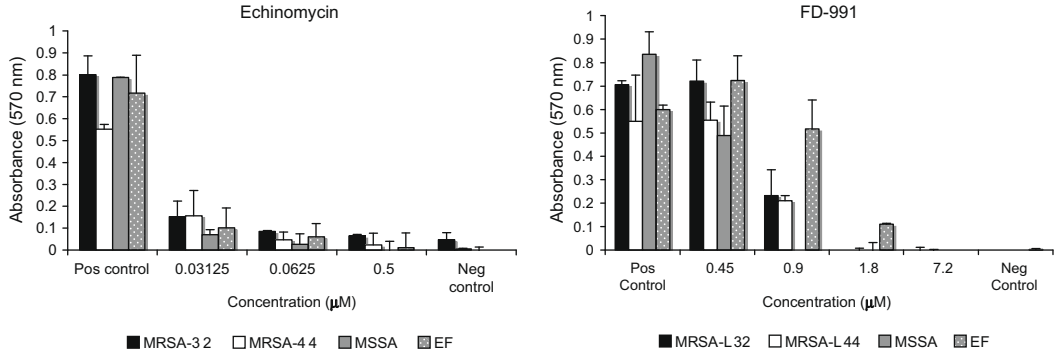

Figure 3. Effects of echinomycin (**1**) and FD-991 (**2**) on biofilm formation by *S. aureus* and *E. faecalis* strains (n = 2).

Table 3
Modified Calgary pin-lid bioassay data

Analog	MRSA-32		MRSA-44		MSSA		EF	
	MBIC (μM)	MBEC (μM)	MBIC (μM)	MBEC (μM)	MBIC (μM)	MBEC (μM)	MBIC (μM)	MBEC (μM)
1	>4	>4	>4	>4	0.03	0.22	0.22	0.22
2	>4	>4	>4	>4	3.6	>4	>4	>4

Antimicrobial susceptibilities of established biofilms were also evaluated using a modified version of the Calgary Biofilm (Pin-lid) Device.[28] This assay determines the antibiotic effects on sessile bacteria seeded from an established biofilm mass. The minimum biofilm inhibitory concentration (MBIC) is defined as no visible growth after incubation for 24 h in the presence of a preformed biofilm and the antibiotic. The minimal biofilm eradication concentration (MBEC) is defined as the minimal concentration of antibiotic that is required to eradicate the CFU/mL growth from the biofilm. The MBIC and MBEC for **1** against MSSA and EF were similar to the respective values for these bacteria when grown planktonically (Table 3). Compound **2** did not demonstrate any notable activity against the panel of isolates in this assay (highest concentration tested = 4 μM).

Echinomycin is a bifunctional DNA intercalator that inserts its quinoxaline rings between 5′-CG nucleotide sequences flanked by A and T base pairs.[29] While this process is entropically driven,[30] additional enthalpic interactions are gained from the alanine carbonyl by formation of a critical hydrogen bond with a guanine 2-amino group in the minor groove.[31,32] Echinomycin belongs to a family of peptide antibiotics that possess variable potencies against Gram-positive, anaerobic, and acid-fast bacteria.[33,34] The results here showed that hydrolysis of one or both of the esters dramatically decreases the antibacterial and cytotoxic potencies, thus indicating the requirement for a bicyclic peptide. Oxidation of the Cys′ sulfur atom of **2** resulted in higher MIC and MBC values against the panel of bacteria. However, **2** was surprisingly more potent than **1** in time-kill studies against *S. aureus*, and demonstrated bactericidal activity against MSSA. This compound is also two-orders of magnitude less toxic than **1** against the human colon cancer cell line. These results suggest that further investigation of the antibacterial properties of **2** may be warranted.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.01.010.

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