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Mutactimycin E, a New Anthracycline Antibiotic with Gram-positive Activity

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Abstract Resistance to currently available antibiotics has become a widely recognized crisis in the medical community. To address this, many companies and researchers are refocusing their attention towards natural products, which have an excellent track record of producing effective antibacterial drugs. The AMRI natural product library was screened for activity against multi-drug resistant *Staphylococcus aureus* (MDRSA). The active samples were counter screened for cytotoxicity against the human hepatocellular carcinoma HepG2 cell line to determine an *in vitro* therapeutic index (*in vitro* TI). Those samples with a high *in vitro* TI were selected for fractionation and dereplication. This led to the discovery of a new anthracycline structure. This metabolite, named mutactimycin E (**1**), exhibited moderate activity against several gram positive organisms. Here we report the isolation, structure elucidation and biological activities of this new compound.

Keywords mutactimycin, anthracycline, antibiotic, *Amycolatopsis*

It has been known for some time that resistance to existing antibiotics is increasing. This is particularly true in hospital settings, but is also found in community-acquired infections. This development, when coupled with the decision by many pharmaceutical companies to abandon antibacterial research in the 1980's and 1990's, resulted in the absence of new antibiotics to combat current resistance mechanisms. In an effort to address this urgent need, we began a screening campaign to identify novel antibiotics from our natural product library. Natural products, particularly those produced by microbial fermentation, were the direct source or inspiration for almost all antibiotics used today and remain the richest source for new antibacterial compound series. Our extensive library consisting of over 280,000 samples was screened for activity against a multi-drug resistant strain of *Staphylococcus aureus* (ATCC 43300). The hits arising out of this assay were then tested against the human hepatocellular carcinoma cell line HepG2 to filter out those samples where activity was the result of general cytotoxicity. The resulting subset of samples possessing selectivity for the bacterial target were then fractionated on an HPLC system employing UV, evaporative light scattering, and MS detectors. The eluted fractions were collected into 96-well microtiter plates and submitted for bioassay. Active compounds were subsequently dereplicated on the basis of MS and UV data.

One of the hits resulting from our efforts originated from the EtOAc extract of *Amycolatopsis* strain 17128. A scaled-up fermentation of the active strain and subsequent purification of the active compound by reverse phase HPLC yielded mutactimycin E (**1**) as an orange solid (Fig. 1). LC/MS data for **1** indicated a molecular weight of 560 Daltons. This piece of information along with the UV spectrum was used to search internal and external databases [1]. Analysis of the hits from this search indicated

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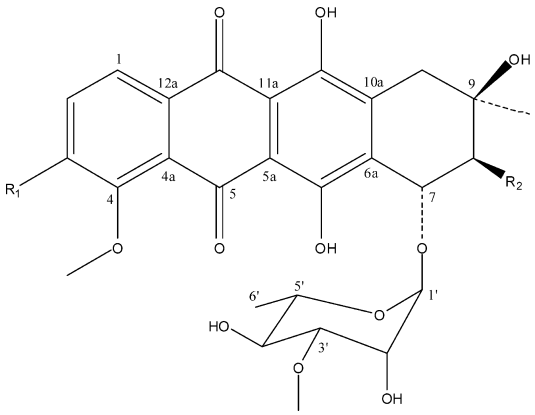

	R_1	R_2
1	Me	OH
Mutactimycin A	Me	H
Mutactimycin D	CH_2OH	H

Fig. 1 Structure of mutactimycin E (**1**).

the compound was related to the mutactimycins [2～5], and possibly was identical to the known metabolite mutactimycin D [6]. However, closer examination of the data suggested **1** was a new member of this class of anthracyclines. HRMS analysis of **1** provided a molecular formula of $C_{28}H_{32}O_{12}$ based on an ion at *m/z* 583.1791 (calcd. 583.1802). A fragment in the LC/MS at *m/z* 401 corresponded to the loss of the sugar moiety. Analysis of the COSY spectrum revealed correlations between the five sugar protons. The observation of the coupling constants (Table 1) indicated that the sugar was a mannopyranose. An HMBC correlation was observed between a methyl group at δ 3.26 (H-3-O-Me) and H-3′ on the sugar thus establishing the position of the methoxy group. Additional examination of the NMR data and comparison with literature values allowed us to identify the sugar moiety as a 6-deoxy-3-*O*-methyl-α-mannopyranoside [3, 4]. An HMBC correlation was observed between the anomeric proton at δ 5.19 (H-1′) and a carbon at δ 74.9 (C-7) (Fig. 2) which indicated the sugar was attached at the C-7 position. Although the molecular weight and UV matched well with mutactimycin D, the absence of an NMR resonance consistent with a CH_2OH group, as expected for mutactimycin D (Fig. 1), confirmed that the structure of **1** was different. Furthermore, an HMBC correlation was seen between the methyl group at δ 1.32 (C-9-Me) and a carbon at δ 73.0 (C-8). This carbon had a corresponding proton signal in the HSQC spectrum which appeared as a doublet at δ 3.67 (H-8). HMBC correlations from the latter to δ 135.7 (C-6a) and δ 70.1 (C-9) and a COSY correlation to a doublet at δ 4.90 (H-7) confirm the presence of a hydroxyl group at C-8. NMR data for the remaining aglycone resonances were consistent with those of other mutactimycins [3, 4]. The stereochemistry at C-7 and C-9 were established as shown based on comparison with literature values [4]. An NOE experiment revealed the relative stereochemistry at C-8 *via* a correlation between H-

Table 1 ^{1}H- and ^{13}C-NMR Data for mutactimycin E (**1**) in DMSO-d_6 (25°C)

Position	δ_H (mult., *J* in Hz)	δ_C
1	7.98 (d, 7.8)	122.6
2	7.78 (d, 7.9)	137.0
3		141.4
4		159.0
4a		125.0
5		186.5
5a		110.0[a]
6		157.8
6a		135.7
7	4.90 (d, 3.8)	74.9
8	3.67 (d, 4.1)	73.0
9		70.1
10	2.63 (d, 18.2)	33.8
	2.77 (d, 18.1)	
10a		137.7
11		155.0
11a		110.7[a]
12		185.7
12a		133.1
3-Me	2.37 (s)	16.3
9-Me	1.32 (s)	26.6
4-O-Me	3.82 (s)	60.7
1′	5.19 (br s)	103.7
2′	3.97 (m)	66.3
3′	3.29 (dd, 9.3, 3.3)	80.2
4′	3.31 (dd, 9.3, 9.3)	70.7
5′	3.80 (dq, 9.2, 6.2)	69.2
6′	1.19 (d, 6.2)	17.9
3′-O-Me	3.26 (s)	56.1
6-OH	13.26 (br s)	
8-OH	4.58 (br s)	
9-OH	4.86 (br s)	
11-OH	14.20 (br s)	
2′-OH	4.81 (d, 4.1)	
4′-OH	4.75 (br s)	

[a] Assignments may be interchanged.

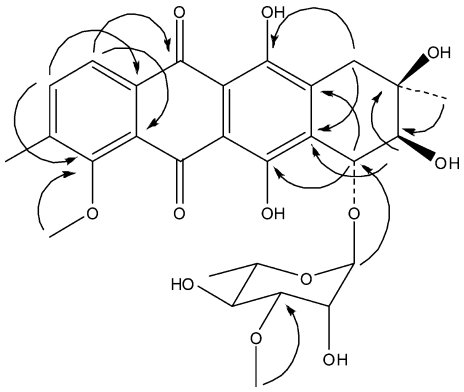

Fig. 2 Key HMBC correlations of **1**.

Table 2 Physico-chemical properties of **1**

Appearance	Orange crystals
Molecular weight	560
Chemical formula	$C_{28}H_{32}O_{12}$
HR FTICR-MS $[M+Na]^+$ (m/z)	Found 583.1791 Calcd 583.1802
UV-VIS λ_{max} (CH_3CN) nm	212, 235, 257, 295, 352, 470, 491, 522
IR ν_{max} (film) cm^{-1}	3406, 2936, 1681, 1614, 1573, 1409, 1257, 1237, 1137, 1102, 1055, 1032, 819
$[\alpha]_D^{22}$ (c 0.002, MeOH)	+78.5°

8 and the methyl group at C-9. Therefore, the structure of **1** was established as mutactimycin E (8-OH mutactimycin A). Its physico-chemical properties are summarized in Table 2.

1 displayed moderate antimicrobial activity against an internal panel of pathogens, which is in good agreement with the bioactivity reported for other mutactimycins [3, 4]. It is also reported that several other members of this group exhibit antiviral activity [3].

A summary of the bioactivities of **1** and antibiotic standards daptomycin [7], linezolid [8], and meropenem [9] against a panel of clinical isolates is shown in Table 3 [10]. **1** does not affect HepG2 cell viability at 128 μg/ml, the highest concentration of compound tested. Furthermore, no cytotoxic effect was observed when **1** was tested against a normal human dermal fibroblasts (NHDF) cell line.

Experimental

General Experimental Procedures

^{1}H- and ^{13}C-NMR as well as COSY, HSQC, and HMBC spectra were recorded using a Bruker DRX 500 NMR spectrometer in DMSO-d_6 at 500 MHz for ^{1}H- and 125 MHz for ^{13}C-NMR, respectively. Mass spectrometry was performed on a Sciex API150 EX single quadrupole with an ionspray ionization source operating in positive mode. High resolution mass spectra (HRMS) were gathered on a Bruker APEX III 47e Fourier Transform (Ion Cyclotron Resonance) Mass Spectrometer at the Mass Spectrometry Center, University of Washington, Seattle, Washington. Infrared data were obtained on a Perkin Elmer 1600 series FTIR.

Semi-preparative HPLC was carried out using a Waters 600 pump connected to a Waters 996 diode-array detector and controlled by Waters Empower software. The purification employed a Phenomenex Luna C_{18} column (10×250 mm; 5.0 μm). Silica gel (230~400 mesh, EM Science) was used for column chromatography. Fractions were collected (~50 ml) and subsequently monitored by TLC and spots were visualized under UV light.

Producing Organism and Fermentation

The *Amycolatopsis* strain 17128 was isolated from a soil sample collected under a rock near Ruby, Arizona. The culture was isolated by spread-plating on Kuster's agar containing 50 μg/ml each of cycloheximide and nystatin, 10 μg/ml each of nalidixic acid and novobiocin and incubating in the dark at 28°C for 10 days. Observed colonies were transferred to and maintained on starch-casein agar plates.

The fermentation procedure was a two-step process in which a suspension of spores and mycelium was inoculated into 250-ml flasks containing 25 ml of a nutrient seed medium having the following composition per liter: 20 g D-glucose, 15 g pharmamedia, 5.0 g yeast extract, 4.0 g $CaCO_3$, 3.0 g $(NH_4)_2(SO_4)$, and 0.03 g $ZnSO_4 \cdot 7H_2O$, adjusted to pH 6.5 prior to autoclaving. After inoculation, the flasks were incubated on a rotary shaker at 250 rpm (2″ throw) and 28°C for 2 days. 1.0 ml aliquots of the seed culture were then used to inoculate one hundred 250-ml flasks containing 30 ml of a production medium with the following composition per liter: 20 g glycerol, 20 g dextrin, 10 g soytone, 3.0 g yeast extract, 2.0 g $(NH_4)_2(SO_4)$, and 2.0 g $CaCO_3$, adjusted to pH 7.0 prior to autoclaving. Following inoculation, the production flasks were incubated on a rotary shaker at 250 rpm and 28°C for 7 days. The fermentation flasks were then harvested, and the

fermentation mixture from each flask was pooled into a single vessel (~3 liters) for extraction with EtOAc.

Extraction and Isolation

Cultures were harvested by extracting with an equal volume of EtOAc. The organic layer was dried over Na_2SO_4 and evaporated to give a dark red solid (1.0 g). A portion of this (800 mg) was dissolved in 200 ml of MeOH and washed with hexane (3×200 ml). The MeOH soluble portion (600 mg) was fractionated over a silica gel column (50 g) using a solvent system of $CHCl_3$ and MeOH (3 : 1). Fractions 2~9 contained the desired product and were combined to yield 300 mg. This material was dissolved in $CHCl_3$ (~5.0 ml) which gave a cloudy solution. MeOH was added dropwise until the solution became clear. After 3 hours at −20°C, a precipitate formed. The latter was recovered and rinsed with hexane to give 80 mg of a dark red solid. The compound was further purified by reversed-phase HPLC using a gradient solvent system of water and CH_3CN each containing 0.05% TFA (95 : 5 to 0 : 100 in 15 minutes; flow 5.0 ml/minute) to yield 40 mg of **1**.

Antibacterial Assays

The agents tested were the natural product-derived antibiotic **1**, daptomycin (DAP), meropenem (MER) and linezolid (LNZ) (Table 3) and vancomycin (data not shown). Organisms used in this study included strains from the ATCC collection and clinical isolates. MICs were determined using the microdilution method described by CLSI [10].

Cell Culture

Human hepatocellular carcinoma HepG2 and NHDF cell lines were purchased from ATCC and Lonza, respectively. The HepG2 cell line was maintained in MEM (minimal essential medium) with 10% heat-inactivated FBS (fetal bovine serum) (Invitrogen), 0.10 mM non-essential amino acids solution (Invitrogen) and 1.0 mM sodium pyruvate (Invitrogen) in a 5% CO_2, 37°C humidified atmosphere. The NHDF cell line was maintained at no more than 16

Table 3 Bioactivities of **1** and selected standards against a panel of clinical isolates

ORGANISM		MIC (μg/ml)			
	PHENOTYPE	**1**	DAP[a]	LNZ[b]	MER[c]
Staphylococcus aureus 100	MSSA[d]	16	1	4	0.25
Staphylococcus aureus 1137	MRSA[e]	8	1	4	>4
Staphylococcus aureus 2012	VISA[f]	8	8	1	>4
Staphylococcus aureus 1725	LRSA[g]	16	1	4	0.25
Staphylococcus aureus 6538	MSSA	1	ND	2	ND
Staphylococcus aureus 43300	MDRSA[h]	8	ND	0.5	ND
Streptococcus pneumoniae 975	PSSP[i]	2	0.25	1	0.015
Streptococcus pneumoniae 940	PRSP[j]	1	0.25	0.25	1
Streptococcus pneumoniae 376	Quin-R[k]	2	0.25	1	0.015
Enterococcus faecium	VRE	8	ND	1	ND
Haemophilus influenzae 1742	ampR[l]	>64	>64	8	0.06
Haemophilius parainfluenzae 2319		>64	>64	16	0.03
Escherichia coli 102	QC strain	>64	>64	>64	0.03
Escherichia coli 2269	ESBL[m]-prod	>64	>64	>64	0.03
Escherichia coli 1411		>64	ND	>64	ND
Klebsiella pneumoniae 2239		>64	>64	>64	0.06
Klebsiella pneumoniae 2262	ampC, MDR[n]	>64	>64	>64	>64
Moraxella catarrhalis 557		8	16	4	≤0.004
Serratia marcescens 1635		>64	>64	>64	0.06
Pseudomonas aeruginosa 1473		>64	>64	>64	4

[a] Daptomycin, [b] Linezolid, [c] Meropenem, [d] Methicillin-susceptible *Staphylococcus aureus*, [e] Methicillin-resistant *Staphylococcus aureus*, [f] Vancomycin-intermediate *Staphylococcus aureus*, [g] Linezolid-resistant methicillin-resistant *Staphylococcus aureus*, [h] Multi-drug resistant *Staphylococcus aureus*, [i] Penicillin-susceptible *Streptococcus pneumoniae*, [j] Penicillin-resistant *Streptococcus pneumoniae*, [k] Quinolone-resistant *Streptococcus pneumoniae*, [l] Ampicillin-resistant, [m] Extended spectrum beta-lactamase, [n] ampC beta-lactamase, multidrug-resistant.

cell passages in a 5% CO_2, 37°C humidified atmosphere using FGM-2BulletKit® (Lonza) based on Lonza's recommendations.

Cytotoxicity Assay

HepG2 and NHDF cells were seeded and cultured in microtiter plates 24 hours prior to the addition of test samples. Compounds were resuspended in DMSO and diluted in assay media for a concentration testing range of 0.6 to 128 μg/ml at a final DMSO concentration of 1.0%. After incubation with the test samples for 48 hours, cell viability was assayed by measuring changes in cellular ATP levels using the CellTiter Glo® Luminescent kit from Promega following the manufacturer's instructions [12, 13]. Cytotoxicity was calculated as a percentage of the DMSO control.

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