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NOTE

5-Hydroxy ericamycin, a new anthraquinone with potent antimicrobial activity

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The departure of many pharmaceutical companies from antibiotic research starting in the 1980s resulted in the absence of new antibiotics to combat the current crisis of increasing microbial resistance to currently available antibiotics. In an effort to address the increasing need for novel antibiotics, AMRI began a screening campaign to identify potent compounds 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. AMRI's extensive library consisting of over 280 000 samples was screened for activity against a multi-drug resistant strain of *Staphylococcus aureus* (ATCC 43 300, Rockville, MD, USA). 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 and determine an *in vitro* therapeutic index (*in vitro* TI). Samples with a high *in vitro* TI were selected for fractionation and dereplication. The resulting subset of samples possessing selectivity for the bacterial target were then fractionated on an HPLC system employing UV, ELSD (evaporative light scattering) and MS detectors. The eluted fractions were collected into 96-well microtiter plates and submitted for bioassay. LC/MS data for the active fractions generated UV spectra and molecular weights, which were used to search internal and external databases.[1] The analysis of the extract from strain 4731, which exhibited excellent activity against several Gram-positive organisms, resulted in the discovery of a new anthraquinone, which was closely related to the known antibiotic ericamycin.[2,3] Here we report the isolation, structure elucidation and biological activities of **1**.

The *Actinoplanes* sp. strain 4731 was isolated from a soil sample collected from grassland near Nanton, in Alberta, Canada. The culture was isolated by a previously described capillary chemotaxis technique,[4] using 0.18% mannitol as a chemo-attractant, spread-plating on water-yeast extract agar plates[5] and incubating in the dark at 28 °C for 10 days. All colonies visible under a dissecting scope were transferred to and purified on starch casein agar plates. Pure cultures were macerated and stored in a 10% glycerol/5% lactose solution at −80 °C. Before fermentation, the culture was streaked from a cryovial onto starch casein agar to verify purity. Fresh culture macerate was prepared from these agar plates after 14 days and used to inoculate the fermentation. Strain 4731 was identified by sequencing of the 16S rRNA gene. Genomic DNA was isolated from a culture grown in tryptic soy broth for 7 days at 28 °C by a phenol:chloroform extraction method.[6] The 16S rRNA gene was amplified using Taq polymerase (Promega, Madison, WI, USA) and the following two primer pairs: 27F, 5′-AGAGTTTGATCMTGGCTCAG-3′; 1115R, 5′-AGGGTTGCGCTCGTTG-3′; and 339F, 5′-CTCCTACGGGAGGCAGCAG-3′; 1429R, 5′-TACGGYTACCTTGTTACGACTT-3′. An NCBI BLAST search of our consensus sequence showed the four closest matches (99% max identity) were all *Actinoplanes* strains.

The fermentation procedure utilized was a two-step process, in which a suspension of culture macerate (mycelium and spores) was inoculated into 250-ml flasks containing 30 ml of a nutrient seed medium having the following composition per liter: 20 g D-glucose, 15 g Pharmamedia (ADM Traders Protein, Lubbock, TX, USA), 5 g yeast extract (Difco, Franklin Lakes, NJ, USA), 4 g $CaCO_3$, 3 g $(NH_4)_2SO_4$ and 0.03 g $ZnSO_4 \bullet 7\ H_2O$, adjusted to pH 6.5 before autoclaving. After inoculation, the flasks were incubated on a rotary shaker at 250 r.p.m. (2″ throw) and 28 °C for 2 days. One milliliter 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: 30 g D-glucose, 10 g maltose, 20 g Quaker oatmeal and 4 g yeast extract, adjusted to pH 7.0 before autoclaving. Following inoculation, the production flasks were incubated on a rotary shaker at 250 r.p.m. and 28 °C for 8 days.

The combined cultures (~3 l) were harvested by extracting with an equal volume of ethyl acetate resulting in a total extract volume

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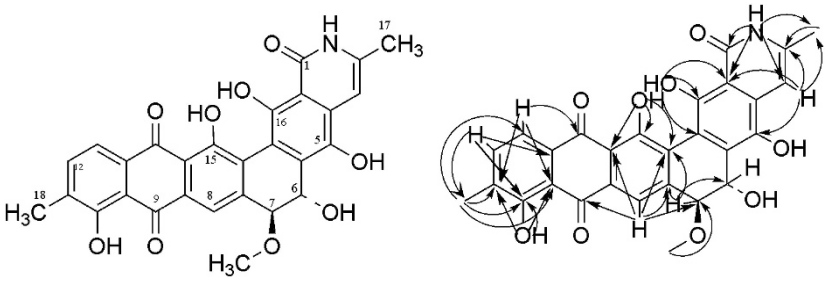

Figure 1 The structure of **1** and its key HMBC correlations.

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

		MIC ($\mu g\,ml^{-1}$)			
Organism	*Phenotype*	***1***	*VAN*[a]	*LNZ*[b]	*MER*[c]
Staphylococcus aureus 43300	MRSA[d]	0.016	2	1	ND
S. aureus 6538	MSSA[e]	0.004	0.5	0.5	0.12
S. aureus 29213	MSSA	0.016	1	4	0.25
S. aureus 1137	MRSA	<0.06	1	4	>4
S. aureus 2012	VISA[f]	<0.06	8	1	>4
S. aureus 1725	LRSA[g]	<0.06	1	4	0.25
S. aureus 2018	VISA[f]	<0.06	8	ND	ND
S. aureus 1651	LRSA[g]	<0.06	1	ND	ND
S. aureus 2144	CA[h], USA 300 strain	<0.06	1	ND	ND
S. epidermis 1597	MSSE[i]	<0.06	2	ND	ND
S. epidermis 1452	MRSE[j]	<0.06	2	ND	ND
S. saprophyticus 495		<0.06	1	ND	ND
Enterococcus faecium 700221	VRE[k]	0.25	>64	1	ND
E. faecalis 846	VRE	<0.06	>64	ND	ND
Streptococcus pneumoniae 940	PRSP[l]	⩽0.06	0.5	0.5	1
S. pneumoniae 376	Quin-R[m]	⩽0.06	0.25	1	0.015
S. pneumoniae 933	MDR[n]	⩽0.06	0.5	ND	ND
S. pyogenes 723		⩽0.06	0. 5	ND	ND
S. agalactiae 2033		⩽0.06	0.5	ND	ND
Haemophilus influenzae 1742	ampR[o]	0.5	>64	8	0.06
H. parainfluenzae 2319		2	>64	16	0.03
Escherichia coli 102	QC strain	4	>64	>64	0.03
E. coli 2269	ESBL[p]-prod	16	>64	>64	0.03
Klebsiella pneumoniae 2239		>64	>64	>64	0.06
K. pneumoniae 2262	ampC[q], MDR	>64	>64	>64	>4
Moraxella catarrhalis 557		⩽0.06	>64	8	⩽0.004
Serratia marcescens 1635		>64	>64	>64	0.12
Pseudomonas aeruginosa 1473		16	ND	>64	ND

Abbreviations: LNZ, linezolid; MER, meropenem; ND, not determined; VAN, vancomycin.
[a]Vancomycin.
[b]Linezolid.
[c]Meropenem.
[d]Methicillin-resistant *Staphylococcus aureus*.
[e]Methicillin-susceptible *Staphylococcus aureus*.
[f]Vancomycin-intermediate *Staphylococcus aureus*.
[g]Linezolid-resistant methicillin-resistant *Staphylococcus aureus*.
[h]Community-acquired.
[i]Methicillin-susceptible *Staphylococcus epidermidis*.
[j]Methicillin-resistant *Staphylococcus epidermidis*.
[k]Vancomycin-resistant Enterococcus.
[l]Penicillin-resistant *Streptococcus pneumonia*.
[m]Quinolone-resistant *Streptococcus pneumonia*.
[n]multidrug-resistant.
[o]Ampicillin-resistant.
[p]Extended spectrum beta-lactamase.
[q]ampC beta-lactamase.

of 2 l. The organic layer was dried over Na_2SO_4 and was concentrated to 80 ml. To the 80 ml solution, 300 ml of petroleum ether was added causing a precipitate to form. The solution was filtered through a Buchner funnel into a 1-l sidearm flask. The collected precipitate was washed with cold methanol to yield 46.3 mg of nearly pure **1**. The latter was further purified by RP-C_{18} 5 μm preparative HPLC (Luna RP-C_{18} column, 250 × 10 mm, Phenomenex, Torrance, CA, USA) using a solvent of H_2O-MeCN each containing 0.05% TFA (95 : 5–0 : 100 in 15 min; 5 ml min^{-1}) to yield 35 mg of **1**.[3]

Compound **1** was obtained as a dark red amorphous solid, optical rotation: $[\alpha]_{589}{}^{22}$ + 560° (c 0.0125, $CHCl_3$), UV (CH_3CN) λ_{max}: 262, 305, 336, 370, 389, 453, 510 nm, IR (film) υ_{max}: 2920, 1687, 1635, 1473, 1421, 1265, 1241, 1224, 1184, 1033, 819, 789, 756 cm^{-1}. Its molecular formula was determined to be $C_{28}H_{21}NO_9$ from the $[M+H]^+$ peak at *m/z* 516.1298 (calculated for $C_{28}H_{22}NO_9$, 516.1295) in the positive ion HR-FTICR-MS spectrum, confirming the addition of an oxygen atom compared with the molecular formula of ericamycin.

A set of ^1H and ^{13}C NMR data were acquired and all ^1H and ^{13}C signals were assigned as shown below. Based on the NMR data, it was quickly established that the substituted anthraquinone skeleton and isoquinoline unit for **1** were very similar to ericamycin.[1] However, the MW was 16 Daltons higher and suggested that **1** contained an additional oxygen atom. Examination of the ^1H NMR spectrum of **1** showed the absence of a singlet at δ 7.19 (H-5), as expected for ericamycin.[3] Analysis of the HMBC spectrum revealed a correlation between the aromatic proton at δ 6.71(H-4) and a carbon at δ 140.2 (C-5). This down field shift of the 5 position carbon from δ 110.1 to δ 140.2,[2] coupled with the loss of the singlet at δ 7.19 (H-5) indicates the substitution of a proton with an oxygen atom. HMBC and HSQC data of the anthraquinone portion of **1** indicated a near perfect match to that reported for ericamycin. Further HMBC correlation of the δ 13.41 (16-OH) yielded the expected upfield shift of δ 152.2 (C-16). The key HMBC connectivities are summarized in Figure 1. It was reported[3] that the pseudodiaxial protons at H-7, O-Me-7 and H-6 were observed as broad singlets, making HMBC correlations from this region difficult, which was also found for **1**. All other chemical shifts support a single hydroxyl substitution of ericamycin at C-5. Therefore, the structure of **1** was established as 5-hydroxy ericamycin. ^1H and ^{13}C NMR chemical shifts of **1** in DMSO-d_6 were following; δ_H (mult., *J* in Hz): 2.31 (Me-17, s, 3H), 2.35 (Me-18, s, 3H), 3.34 (O-Me-7, br s, 3H), 4.46 (H-7, d, 4.4), 5.23 (H-6, br s), 5.24 (OH-6, br s), 6.71 (H-4, s), 7.73 (H-12, d, 7.8), 7.76 (H-13, d, 7.8), 7.85 (H-8, s), 8.72 (OH-5, br s, 3H), 11.90 (NH-2, s), 12.97 (OH-10, s), 13.41 (OH-16, s), and 13.42 (OH-15, s); δ_c 15.5 (Me-18), 18.7 (Me-17), 56.4 (O-Me-7), 62.1 (C-6), 79.9 (C-7), 100.2 (C-4), 109.7 (C-16a), 112.2 (C-15b), 114.7 (C-9a), 115.7 (C-14a), 118.6 (C-13), 120.4 (C-8), 129.6 (C-15a), 129.9[a] (C-4a), 130.2[a] (C-5a), 130.5 (C-8a), 131.2 (C-13a), 134.7 (C-11), 137.1 (C-12), 138.5 (C-3), 140.2 (C-5), 142.9 (C-7a), 152.8 (C-16), 159.9 (C-10), 159.9 (C-15), 166.4 (C-1), 187.4 (C-9) and 187.6 (C-14) ([a]assignments may be interchanged).

The agents tested were **1**, vancomycin, meropenem and linezolid. Organisms used in this study included strains from the ATCC collection and clinical isolates. MICs were determined using the microdilution method described by CLSI.[7] Compound **1** displayed excellent antimicrobial activity, as low as < 0.06 μg ml^{-1}, against several pathogens including many resistant strains. Its activities compared very favorably with vancomycin[8] and linezolid,[9] which are currently on the market as antibiotics targeting Gram-positive microorganisms. However, **1** showed less activity against some Gram-negative pathogens than meropenem.[10] Ericamycin was reported to have much lower activity against several *Staphylococcus* strains, *Pseudomonas aeruginosa*, *E. coli* and other pathogens.[2] A summary of the bioactivities of **1** and the antibiotic standards against a panel of clinical isolates is shown in Table 1 (Data were generated in house and by Micromyx, LLC (Kalamazoo, MI, USA)). The cytotoxic concentration required to inhibit the growth of 90% of HepG2 cells (CC_{90}) was measured for both compounds, and **1** affected HepG2 cell viability after 48 h at 4.7 μg ml^{-1} indicating some toxicity against mammalian cells. **1** was also tested against a normal human dermal fibroblast cell line and almost identical cytotoxic effects were observed with a CC_{90} of 4.7 μg ml^{-1}.

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