Notes

Citreamicins with Potent Gram-Positive Activity

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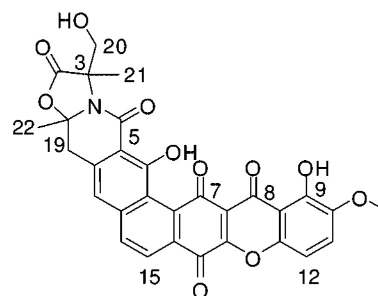
Two new xanthone antibiotics, citreamicin δ (**1**) and ε (**2**), with potent activity against Gram-positive pathogens including multidrug-resistant *Staphylococcus aureus* (MDRSA) were discovered. Compounds **1** and **2** exhibited MIC values < 1 μg/mL versus a number of resistant strains. The compounds were obtained from EtOAc extracts of *Streptomyces vinaceus* and were purified by countercurrent chromatography and reversed-phase HPLC. Their structures were elucidated using primarily NMR and mass spectroscopy.

It has been known for some time that microbial resistance to existing antibiotics is increasing. This is particularly true in hospital settings, but is also found in community-acquired infections. When coupled with the decision by many pharmaceutical companies to abandon antibacterial research in the 1980s and 1990s, this development resulted in the absence of new antibiotics to combat current resistance mechanisms. In an effort to address this urgent need, AMRI began a screening campaign to identify novel antibiotics from our natural products resources. Natural products were the direct source or inspiration for virtually all antibiotics for many years and remain the richest source for discovery of new antibiotics therapies. Our extensive library consisting of over 280 000 samples was screened for activity against multidrug-resistant *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. This also served to establish an *in vitro* therapeutic index (*in vitro* TI). The resulting subset of samples possessing selectivity for the bacterial target were then fractionated on an HPLC system employing UV, ELSD, 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 an EtOAc extract of an actinomycete strain identified as *Streptomyces vinaceus* according to its 16S rRNA gene (99% sequence similarity). LC/MS data for the active components returned UV spectra and molecular weights that yielded no matches in the internal and external databases.[1] This triggered a scale-up fermentation of the active strain to a 3 L scale, extraction of the fermentation broth with EtOAc, and subsequent purification of the active compounds. The EtOAc extract was fractionated by countercurrent partition chromatography (CPC). This resulted in two active peaks, which were further purified using reversed-phase HPLC to yield **1** and **2**.

Compound **1** gave a molecular formula of $C_{30}H_{21}NO_{11}$ based on an exact mass of 594.1001 for the M + Na peak (calcd 594.1012). The ^{1}H NMR spectrum revealed the presence of two phenolic protons, five aromatic protons, one methoxy group, two quaternary methyl groups, and two CH_2 groups. This left one proton unaccounted for, which was presumed to be exchangeable. The ^{13}C NMR spectrum revealed five signals between δ_C180 and 165,



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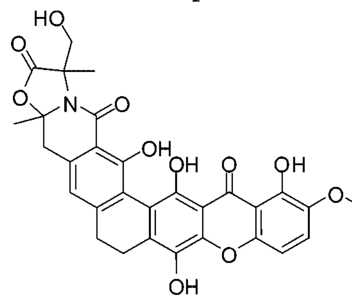

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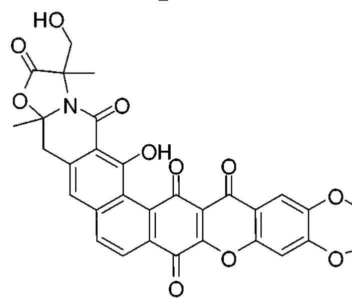

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suggesting five conjugated carbonyl groups; five signals between δ_C 162 and 144, suggesting aromatic carbons attached to oxygen; 13 peaks between δ_C 142 and 107, indicating multiple aromatic rings; and seven nonaromatic signals between δ_C 92 and 19. The presence of two sharp singlets at δ_H 12.39 and 13.55 indicated the two OH protons hydrogen bonded to two carbonyl groups. The proton signal at δ_H 12.39 showed HMBC correlations to three

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carbons at δ_C 112.6, 150.2, and 144.8. The latter also had a correlation to a singlet at δ_H 3.96, indicating the point of attachment for the methoxy group. A proton signal at δ_H 7.34 gave a COSY correlation to a proton at δ_H 7.15 and HMBC correlations to carbons at δ_C 144.8, 150.2, and 148.2. Collectively, this data allowed for the construction of the F and G rings. The second OH proton at δ_H 13.55 gave HMBC correlations to resonances at δ_C 107.4, 162.2, and 119.2. A broad singlet at δ_H 7.15 in the proton spectrum shared cross-peaks with the resonances at δ_C 107.4 and 119.2, suggesting it was situated *para* to the OH group. This proton also displayed a correlation with a CH_2 group at δ_C 40.4. Another proton at δ_H 7.90 correlated to the ^{13}C NMR resonances at δ_C 119.2 and 117.5. This doublet also showed a COSY correlation to a proton resonance at δ_H 8.19, which in turn correlated with ^{13}C NMR signals at δ_C 137.6, 140.8, and 177.6. Together, these correlations provided justification for the partial assembly of the B, C, and D rings. Finally, HMBC correlations from the methyl group at δ_H 1.69 to carbons at δ_C 172.8 and 63.4 and from the methyl group at δ_H 1.67 to carbons at δ_C 40.4 and 92.9 provided evidence for assignment of the A ring. Three carbons at δ_C 180.3, 119.9, and 154.8 remained unaccounted for in the structure. These were assigned to the remaining pieces of the E ring and completed the structure of **1** as shown.

Compound **2** had a molecular weight of 575, indicating the presence of four additional protons compared to **1**. This was proven by the HRMS for this compound showing an exact mass of 576.1525 for the $M + H^+$ ion corresponding to a formula of $C_{30}H_{25}NO_{11}$. The ^{1}H NMR spectrum of **2** looked very similar to that of **1** with two important exceptions. First, it indicated the presence of four aliphatic protons not seen in **1**. Also evident was the disappearance of two aromatic protons in **2** when compared to **1**. Therefore, it was clear that two of the additional protons were the result of hydrogenation of a double bond. The location of the other two additional protons was inferred by a color change for **2** from red to yellow relative to **1**. This suggested the quinone moiety had been reduced to the hydroquinone. Analysis of the ^{13}C NMR spectrum supported this hypothesis by revealing the substitution of two phenolic resonances for the two unsaturated ketone signals seen in **1**. This allowed the structure of **2** to be assigned as shown. The NMR data for **1** and **2** are summarized in Table 1.

A NOESY spectrum was obtained in an effort to deduce the relative stereochemistry at positions 3 and 19. The data revealed a cross-peak between the two methyl groups at δ_H 1.56 and 1.60, indicating the likelihood that they were on the same side of the molecule. However, given the small difference in chemical shifts between these two resonances, these results should be viewed with caution. Furthermore, the absence of a correlation between the methyl group at position 19 and the methylene at position 3 supports this assignment. Related compounds reported in the literature do not report the stereochemistry at these positions, making direct comparisons impossible.[2]

Searching the literature for related compounds led to the closely related citreamicin series of structures.[2] In particular, citreamicin η (**3**) was very similar to **1**, with the only differences being the relocation and demethylation of one methoxy group. Citreamicin η was reported to have *in vitro* MIC values of <0.015 μg/mL against several Gram-positive strains. Likewise, **1** and **2** displayed exceptional activity, as low as <0.06 μg/mL, against several pathogens including many resistant strains.[3] Their activities compared very favorably with daptomycin[4] and linezolid,[5] which are currently on the market as Gram-positive antibiotics. However, they did show less activity against Gram-negative pathogens than meropenem.[6] A summary of the bioactivities of **1** and **2** and these standards against a panel of clinical isolates is shown in Table 2. The cytotoxic concentration required to inhibit the growth of 90% of HepG2 cells (CC_{90}) was measured for both compounds, and **1** and **2** affect HepG2 cell viability after 48 h at 2.4 and 1 μg/mL, respectively, indicating some toxicity against mammalian cells.

Table 1. ^{1}H and ^{13}C NMR Data for **1** and **2**[a]

	1		**2**	
position	δ_c	δ_H (mult., J in Hz)	δ_c	δ_H (mult., J in Hz)
2	172.8		173.3	
3	65.1		65.4	
5	165.6		165.8	
5a	107.4		108.9	
6	162.2		147.1	
6a	119.2		119.2	
6b	137.6		113.2	
7	180.3		149.8	
7a	119.9		105.6	
8	175.0		186.1	
8a	112.6		107.6	
9	150.2		149.0	
10	144.8		142.5	
11	120.0	7.34 (d, 9.1)	121.8	7.56 (d, 9.17)
12	107.4	7.15 (d, 8.9)	106.0	7.05 (d, 9.1)
12a	148.2		148.8	
13a	154.8		144.4	
14	177.6		138.6	
14a	129.9		132.6	
15	124.4	8.19 (d, 8.5)	23.4	2.18 (m)
16	132.5	7.90 (d, 8.5)	29.4	2.83 (m)
16a	140.8		138.7	
17	117.5	7.15 (s)	117.5	6.82 (s)
17a	135.4		134.9	
18	40.4	3.36 (d, 14.8)	39.7	3.40 (m)
		3.49 (d, 15.7)		3.50 (d, 14.7)
19	92.9		93.5	
20	63.4	3.89 (d, 11.2)	63.3	3.65 (d, 10.0)
		4.53 (d, 11.2)		4.08 (d, 10.5)
21	19.1	1.69 (s)	18.4	1.56 (s)
22	26.8	1.67 (s)	24.9	1.60 (s)
OMe	56.8	3.96 (s)	56.6	3.83 (s)
6-OH		13.55 (br s)		12.54 (br s)
7-OH				11.83 (br s)
9-OH		12.39 (br s)		11.80 (br s)
14-OH				9.26 (br s)

[a] 500 and 125 MHz, respectively; measured in DMSO-d_6.

Compounds **1** and **2** were also tested against a normal human dermal fibroblast (NHDF) cell line, and similar cytotoxic effects were observed with a CC_{90} of 0.3 and 0.1 μg/mL, respectively.

Experimental Section

General Experimental Procedures. ^{1}H and ^{13}C NMR as well as COSY, NOESY, 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. Infrared data were obtained on a Perkin-Elmer 1600 series FTIR. Countercurrent chromatography was accomplished with a PC Inc. instrument using a 400 mL coil. Semipreparative 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 μm). Mass spectrometry was performed on a Sciex API150 EX single quadrupole with an ionspray ionization source operating in positive mode. High-resolution mass spectra were gathered on a Bruker APEX III 47e Fourier transform (ion cyclotron resonance) mass spectrometer [FT(ICR)]MS at the Mass Spectrometry Center, University of Washington, Seattle, WA.

Isolation of Producing Organism and Its Phylogenetic Affiliation. *Streptomyces vinaceus* strain AMRI-45379 was isolated in our laboratory from a soil sample collected from the roots of a cedar tree at the water line of the Sabine River in Texas. The culture was isolated by spread-plating on trehalose asparagine (Sigma) agar supplemented with 50 μg/mL cycloheximide, 10 μg/mL nalidixic acid, 50 μg/mL nystatin, and 25 μg/mL rifamycin B (all from Sigma) and incubated in the dark at 28 °C for 10 days. Observed colonies were transferred to and maintained on starch-casein agar plates and used to start the fermentation. For DNA extraction, the strain was grown on trypticase soy broth (TSB, Sigma) for 7 days at 28 °C, and the total genomic DNA preparation was carried out following a literature procedure.[7] PCR

Table 2. Bioactivities of **1** and **2** and Selected Standards against a Panel of Clinical Isolates

organism	phenotype	MIC (μg/mL)				
		1	**2**	DAP[a]	LNZ[b]	MER[c]
Staphylococcus aureus 43300	MRSA[d]	0.5	0.13	ND	1	ND
Staphylococcus aureus 6538	MSSA[e]	≤0.5	0.13	ND	0.5	ND
Staphylococcus aureus 29213	MSSA	1	0.12	1	4	0.25
Staphylococcus aureus 1137	MRSA	2	0.25	1	4	>4
Staphylococcus aureus 2012	VISA[f]	2	0.25	8	1	>4
Staphylococcus aureus 1725	LRSA[g]	1	0.12	1	4	0.25
Enterococcus faecium 700221	VRE	≤0.25	0.5	ND	ND	ND
Streptococcus pneumoniae 975	PSSP[h]	≤0.06	0.25	0.25	1	0.015
Streptococcus pneumoniae 940	PRSP[i]	≤0.06	0.12	0.25	0.25	1
Streptococcus pneumoniae 376	Quin-R[j]	≤0.06	0.25	0.25	1	0.015
Haemophilus influenzae 1742	ampR[k]	1	>32	>64	8	0.06
Hemophilius parainfluenzae 2319		1	>32	>64	16	0.03
Escherichia coli 1411		>128	>64	ND	ND	ND
Escherichia coli 25922	QC strain	>64	>32	>64	>64	0.03
Escherichia coli 2269	ESBL[l]-prod	>64	>32	>64	>64	0.03
Klebsiella pneumoniae 2239		>64	>32	>64	>64	0.06
Klebsiella pneumoniae 2262	ampC,MDR[m]	>64	>32	>64	>64	>64
Moraxella catarrhalis 557		4	0.5	16	4	≤0.004
Serratia marcescens 1635		>64	>32	>64	>64	0.06
Pseudomonas aeruginosa 1473		>64	>32	>64	>64	4

[a] Daptomycin. [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] Penicillin-susceptible *Streptococcus pneumoniae.* [i] Penicillin-resistant *Streptococcus pneumoniae.* [j] Quinolone-resistant *Streptococcus pneumoniae.* [k] Ampicillin-resistant. [l] Extended spectrum beta-lactamase. [m] ampC beta-lactamase, multidrug-resistant.

amplification of the 16S rRNA gene of the antibiotic producing strain was performed using two primers: StrepF, 5′-ACGTGTGCAGCCCAA-GACA-3′; StrepR, 5′-ACAAGCCCTGGAAACGGGGT-3′ (IDT).[8] The PCR mixture consisted of 30 pmol of each primer, 100 ng of chromosomal DNA, 200 μM dNTPs (Invitrogen), and 2.5 units of Taq polymerase (Invitrogen) in 50 μL of polymerase buffer. The amplification was carried out for 30 cycles at 94 °C for 1 min, annealing at 53 °C for 1 min, and extension at 72 °C for 2 min. The PCR reaction mixture was analyzed by agarose gel electrophoresis, and the remnant mixture was purified using QIA quick PCR purification reagents (Qiagen). 16S rRNA gene was sequenced on both strands by ACGT, Inc.[9] The Blast program (www.ncbi.nlm.nih.gov/blst) was used to assess the DNA similarities. Multiple sequence alignments were performed using BioEdit software.[10]

Fermentation. The fermentation procedure utilized 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 of D-glucose (Mallinckrodt), 15 g of pharmamedia (Traders protein), 5 g of yeast extract (Difco), 4 g of $CaCO_3$ (Sigma), 3 g of $(NH_4)_2(SO_4)$ (Mallinckrodt), and 0.03 g of $ZnSO_4 \cdot 7H_2O$ (Sigma), adjusted to pH 6.5 prior to autoclaving. After inoculation, the flasks were incubated on a rotary shaker at 250 rpm (2 in. throw) and 28 °C for 2 days. Aliquots (1 mL) 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 of glycerol (Sigma), 20 g of pharmamedia (Trader's Protein), 20 g of dry yeast (Red Star), 21.8 g of KH_2PO_4 (Mallinckrodt), 5.67 g of Na_2HPO_4 (Sigma), and 5 g of $MgCl_2 \cdot 6H_2O$ (Sigma), 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.

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 (0.8 g). A portion of this (100 mg) was taken up in a mixture of solvents used for countercurrent chromatography. A solvent system of hexane/EtOAc/MeOH:H_2O (1:1:1:1) was employed. The instrument was run in normal phase for 1 h at a flow rate of 6 mL/min followed by 1 h in reversed-phase mode at the same flow rate. Two peaks associated with fractions 13−19 and 20−26 showed strong activity. These fractions were combined to yield 4.2 and 4.3 mg, respectively. The pooled fractions were further purified by reversed-phase HPLC on a Phenomenex Luna C_{18} column (10 × 250 mm, 5 μm) using CH_3CN and H_2O each containing 5 mM NH_4OAc. The compounds were eluted using a gradient of 50% CH_3CN going to 100% over 7.5 min to give 2.3 mg of **1** and 2.9 mg of **2**.

Citreamicin δ (1): dark red solid; $[\alpha]_D$ +50 (*c* 0.001, DMSO); UV (CH_3CN) λ_{max} 228, 252, 332, 432 nm; IR ν_{max} (film) 3020, 2927, 1796, 1695, 1623, 1429, 1276, 1205, 1182, 1140, 1027, 956, 802, and 719 cm^{-1}; ^{1}H and ^{13}C NMR data (DMSO-d_6), Table 1; ESMS *m/z* 572 (M + H)$^+$; HR-FTICR-MS [M + Na]$^+$ *m/z* 594.1001 [calcd for $C_{30}H_{21}NO_{11}Na^+$, 594.1012].

Citreamicin ϵ (2): yellow solid; $[\alpha]_D$ −49 (*c* 0.001, MeOH); UV (CH_3CN) λ_{max} 236, 274, 347, 437 nm; IR ν_{max} (film) 3021, 2928, 1795, 1695, 1623, 1429, 1277, 1205, 1181, 1140, 1026, 957, 802, and 719 cm^{-1}; ^{1}H and ^{13}C NMR data (DMSO-d_6), Table 1; ESMS *m/z* 576 (M + H)$^+$; HR-FTICR-MS [M + H]$^+$ *m/z* 576.1525 [calcd for $C_{30}H_{26}NO_{11}$, 576.1506].

Antibacterial Assays. The agents tested were the natural product-derived antibiotics **1** and **2**, linezolid, vancomycin[11] (data not shown), daptomycin, and meropenem. Organisms used in this study included strains from the ATCC collection and clinical isolates. Minimum inhibitory concentration values (MICs) were determined using the microdilution method described by CLSI.[12]

Cell Culture. Human hepatocellular carcinoma (HepG2) and normal human dermal fibroblast (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 nonessential amino acids solution (Invitrogen), and 1 mM sodium pyruvate (Invitrogen) in a 5% CO_2, 37 °C humidified atmosphere. The NHDF cell line was maintained at no more than 16 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 h 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%. After incubation with the test samples for 48 h, cell viability was assayed by measuring changes in cellular ATP levels using the CellTiter-Glo luminescent kit from Promega following the manufacturer's instructions.[13,14] Cytotoxicity was calculated as a percentage of the DMSO control.

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