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Feng Cao
Department of Veterans Affairs Medical Center - St. Louis

Cari Orth
Department of Veterans Affairs Medical Center - St. Louis

Maureen J. Donlin
Saint Louis University School of Medicine

Patrick Adegboyega
Department of Veterans Affairs Medical Center - St. Louis

Marvin J. Meyers
Saint Louis University

See next page for additional authors

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Authors

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Synthesis and Evaluation of Troponoids as a New Class of Antibiotics

Feng Cao,*,[†] Cari Orth,[†] Maureen J. Donlin,[‡] Patrick Adegboyega,[†] Marvin J. Meyers,[§] Ryan P. Murelli,[‖,⊥] Mohamed Elagawany,[#,¶] Bahaa Elgendy,[#,∇] and John E. Tavis[○]

[†]John Cochran Division, Department of Veterans Affairs Medical Center, 915 North Grand Blvd., St. Louis, Missouri 63106, United States

[‡]Edward A. Doisy Department of Biochemistry and Molecular Biology, Saint Louis University School of Medicine, St. Louis, Missouri 63104, United States

[§]Department of Chemistry, Saint Louis University, St. Louis, Missouri 63104, United States

[‖]Department of Chemistry, Brooklyn College, The City University of New York, Brooklyn, New York 11210, United States

[⊥]PhD Program in Chemistry, The Graduate Center of The City University of New York, New York 10016, United States

[#]Center for Clinical Pharmacology, Washington University School of Medicine and St. Louis College of Pharmacy, St. Louis, Missouri 63110, United States

[¶]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Damanhour University, Damanhour 31111, Egypt

[∇]Chemistry Department, Faculty of Science, Benha University, Benha 13518, Egypt

[○]Department of Molecular Microbiology and Immunology, The Saint Louis University Liver Center, Saint Louis University School of Medicine, St. Louis, Missouri 63104, United States

S *Supporting Information*

ABSTRACT: Novel antibiotics are urgently needed. The troponoids [tropones, tropolones, and α-hydroxytropolones (α-HT)] can have anti-bacterial activity. We synthesized or purchased 92 troponoids and evaluated their antibacterial activities against *Staphylococcus aureus*, *Escherichia coli*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*. Preliminary hits were assessed for minimum inhibitory concentrations (MIC_{80}) and cytotoxicity (CC_{50}) against human hepatoma cells. Sixteen troponoids inhibited *S. aureus*/*E. coli*/*A. baumannii* growth by ≥80% growth at <30 μM with CC_{50} values >50 μM. Two selected tropolones (**63** and **285**) inhibited 18 methicillin-resistant *S. aureus* (MRSA) strains with similar MIC_{80} values as against a reference strain. Two selected thiotropolones (**284** and **363**) inhibited multidrug-resistant (MDR) *E. coli* with MIC_{80} ≤30 μM. One α-HT (**261**) inhibited MDR-*A. baumannii* with MIC_{80} ≤30 μM. This study opens new avenues for development of novel troponoid antibiotics to address the critical need to combat MDR bacterial infections.
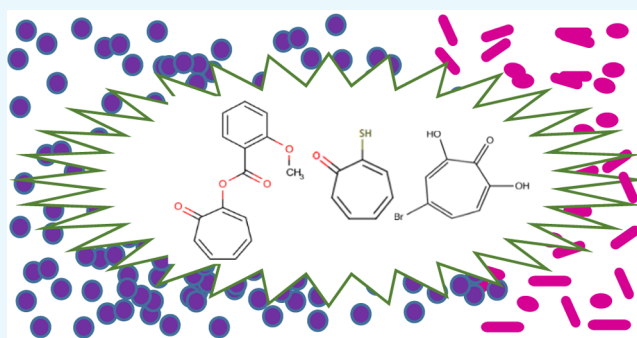
INTRODUCTION

The emergence of antimicrobial-resistant bacteria is a rapidly growing concern for public health. The economic cost of bacterial resistance is estimated to be around $55 billion annually in the United States alone.[1] In February 2017, the World Health Organization (WHO) announced that the highest priority organisms for development of new antibiotics are carbapenem-resistant *Acinetobacter*, *Pseudomonas aeruginosa*, and the Enterobacteriaceae. The first two second-priority organisms are vancomycin-resistant *Enterococcus faecium* and methicillin-resistant, vancomycin intermediate and resistant *Staphylococcus aureus*. The discovery and development of novel antibiotic compounds has been slow. Resistant bacteria spread and cause infections at increasing rates, and thus there is an urgent need to develop novel classes of potent antibiotics.[2,3] In addition, most new antibiotics are derivatives of existing drugs; thus, bacterial targets have already been under strong selection to develop resistance.

Troponoid compounds include the tropones, tropolones, and hydroxytropolones and their derivatives. All of them have a seven-carbon ring and possesses a nonbenzenoid aromatic character.[4] Tropone (2,4,6-cycloheptatrien-1-one) has a ketone group on the troponoid ring. Tropolone (2-hydroxy-2,4,6-cycloheptatrien-1-one) has an alcohol (or an enol including the double bond) group next to the ketone. α-

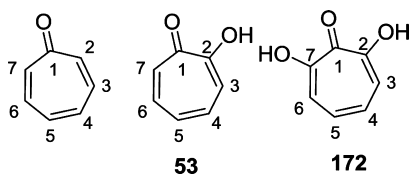Hydroxytropolone (α-HT) has an additional alcohol group on C7 of the troponoid ring (Figure 1).

Figure 1. Structures of (A) tropone, (B) tropolone, and (C) α-HT. Structures for all compounds tested are in Figure S1.

Tropolone derivatives can have antibacterial,[5−9] antiviral,[10−14] antifungal properties,[15] anti-tumor, anti-inflammatory, antioxidant and insecticidal.[16,17] Because troponoids have high pharmacological activity, development of convenient methods of synthesis of their new derivatives and the search among the derivatives for molecules with antibacterial activity against drug resistant bacteria are important. Recently, Dr. Ryan Murelli and coworkers pioneered a novel approach to generate polysubstituted α-HTs from readily available precursor compounds,[18] and Dr. Bahaa Elgendy and coworkers explored the synthesis of novel thiotropolones. Together, these provided us the unique opportunity to evaluate the anti-microbial activities of a wide range of chemically diverse troponoids. Here, 92 natural and synthetic troponoids were screened for inhibition of bacterial growth to assess whether they may be attractive candidates for development into novel antibiotics.

■ RESULTS

Primary Inhibitor Screening. We measured the effect of the troponoids on bacterial growth to test whether they had antibiotic activity. Among the 92 troponoids tested, 18 are tropolones, 26 are tropones and 48 are α-HT. In the initial qualitative screening, the 92 compounds were each tested at 5.8, 20.4, and 71.4 μM. We screened against *Escherichia coli* (ATCC 35218), *Staphylococcus saprophyticus* (ATCC BAA-750), *Acinetobacter baumannii* (Ab1, from a patient), and *P. aeruginosa* (ATCC 27853). The compounds were diluted in cation-adjusted Mueller-Hinton II broth (CAMHB), and bacteria from overnight cultures were added to the diluted compounds in a 96-well plate (5×10^5 CFU/mL inoculum for each well). After 16−24 h incubation at 35 ± 2 °C, the turbidity in the cultures was read at 630 nM in a microplate reader. The percentages of compounds that suppressed bacterial growth by ≥80% relative to vehicle control cultures at the screening concentrations are shown for each bacterial species in Table 1: 9.8 and 8.7% of the compounds inhibited *S. saprophyticus* and *E. coli* growth at 15.2 μM, but at the highest concentration of 71.4 μM, only 20.4% compounds inhibited *A. baumannii*. None of the compounds inhibited *P. aeruginosa* growth at 71.4 μM. All results from the full set of 92 troponoids are in shown Table S1.

Table 1. Percentage of Compounds That Inhibited Bacteria Growth ≥80% Compared to Vehicle-Treatment Control

organism	compounds concentration (μM)	5.8	20.4	71.4
S. saprophyticus	percentage of compounds (%)	0	9.8	39.1
E. coli	percentage of compounds (%)	0	8.7	20.7
A. baumannii	percentage of compounds (%)	0	0	15.2
P. aeruginosa	percentage of compounds (%)	0	0	0

MIC$_{80}$ and CC$_{50}$ Measurement for Troponoids. The minimal inhibitory concentration 80% (MIC$_{80}$) and cytotoxic 50% (CC$_{50}$) values were measured for compounds that inhibited *S. saprophyticus* growth by ≥80% at 20.4 μM, and also those that demonstrated ≥80% inhibition of *E. coli* and *A. baumannii* growth at 71.4 μM in the preliminary screening (Table 2). An overnight bacterial culture was adjusted to 5×10^5 CFU/mL and added to 1.5-fold serially diluted compounds, and turbidity was measured after incubation for 16−24 h. The bacteria (*E. coli*, *A. baumannii*, and *P. aeruginosa*) used in MIC$_{80}$ measurements were the same as for the primary screening, but for *Staphylococcus*, we shifted to *S. aureus* (ATCC 29213) because *S. aureus* is a common pathogen in the Staphylococcaceae family, whereas *S. saprophyticus* is a commensal member of the normal human flora. Cytotoxic CC$_{50}$ values were measured in HepDES19 cells, a HepG2-derived human hepatoblastoma cell line[19] because the liver is a common site of drug toxicity. Serially diluted troponoid compounds were added to HepDES19 cells in a final concentration of dimethyl sulfoxide (DMSO) of 1%. After 3 days of incubation, MTS reagent was added to cells and the cells were incubated for 90 min prior to reading absorbance at 480 nm, and CC$_{50}$ values were calculated by nonlinear curve fitting. Therapeutic index (TI) values, the ratio of the amount of a compound that causes 50% toxicity to the amount that causes 80% efficacy (CC$_{50}$/MIC$_{80}$), were also calculated.

Table 2 shows the MIC$_{80}$, CC$_{50}$, and TI values and the structures of compounds that inhibited ≥80% growth of *S. aureus* at <20 μM, and *E. coli* and *A. baumannii* at <30 μM with CC$_{50}$ >50 μM. Table S1 shows MIC$_{80}$ and CC$_{50}$ values for all 92 troponoids.

Among the 18 tropolones that inhibited *S. aureus*, **53**, **54**, and **338** inhibited growth by >80% at <20 μM with CC$_{50}$s >50 μM and TI values of 8.5, 6.0, and 5.1, respectively. Compound **350** inhibited by >80% at 8.8 μM, but its CC$_{50}$ was less than 50 μM (46 μM). The rest of the tropolones, **47** to **50**, **52**, **55**, **195**, **340** to **345**, and **349** had moderate substitutions on the tropone ring and had decreased or no activity. The 26 tropones tested were all variants of **53** with modifications to the tropolone hydroxyl, and they had variable activities. The −OH was changed to a chlorine in **57**, to an aniline in **60**, and to a sulfonyl ester in **61**. All three had MIC$_{80}$ >100 μM. However, in **363**, the oxygen of the hydroxyl group was changed to sulfur, and activity was only slightly decreased. Inhibition by several benzoylated variants (**62**, **63**, **282**, **283**, **284**, **285**, **348**, and **364**) was similar to that of **53**. However, two benzoylated variants (**61** and **346**) lost all activity, probably because of sulfonyl ester replacement. When the thioester in **284** was changed to a thioether in **365**, all inhibitory activity was lost. For the 48 α-HT compounds, six compounds with appendages on the troponoid ring (**46**, **114**, **120**, **146**, **261**, and **262**) inhibited *S. aureus* at <20 μM. In contrast, **172**, which has no substitutions, had an MIC of 66.7 μM.

Regarding the antibacterial activity of troponoids on Gram-negative rods, including *E. coli*, *Acinetobacter banmannii* and *P. aeruginosa*, four tropones (**284**, **363**, **364** and **680**) and two α-HT (**261** and **310**) inhibited *E. coli* growth by >80% at <30 μM with CC$_{50}$ values >50 μM. Only two α-HTs (**261** and **310**) could inhibit *A. baumannii* by >80% at <30 μM with CC$_{50}$s >50 μM. None of the compounds inhibited *P. aeruginosa* at 71.4 μM, the highest concentration employed.

Inhibition of Multidrug-Resistant *S. aureus* Strains by Compounds 63 and 285. Next, we selected the top two

Table 2. MIC$_{80}$ and TI Values of *E. coli, S. aureus, A. baumannii,* and *P. aeruginosa* and CC$_{50}$ for Selected Compounds
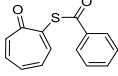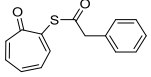
Compound Number	Compound Structure	MIC$_{80}$ (μM) S. aureus (ATCC29213)	E. coli (ATCC35218)	A. baumannii (From a patient)	P. aeruginosa (ATCC27853)	CC$_{50}$ (μM)
Tropolone derivatives						
53		11.7 (8.5)[a]	-	-	-	>100
54		16.1 (6.0)	70.4 (1.4)	44.4 (2.2)	-	96
338		19.8 (5.1)	-	-	-	>100
Tropone derivatives						
51		16.5 (>6.1)	-	-	-	>100
62		19.8 (5.0)	-	83.4 (1.2)	-	99
63		13.2 (7.6)	-	66.7 (1.5)	-	100
282		18.2 (5.3)	-	-	-	97
283		15.4 (6.1)	-	-	-	94.6
284		13.2 (6.9)	24.1 (3.8)	55.6 (1.6)	-	91
285		8.8 (11.4)	-	44.4 (2.3)	-	100
348		8.8 (11.4)	-	44.4 (2.3)	-	100

Table 2. continued

Compound Number	Compound Structure	MIC$_{80}$ (μM) S. aureus (ATCC29213)	E. coli (ATCC35218)	A. baumannii (From a patient)	P. aeruginosa (ATCC27853)	CC$_{50}$ (μM)
363		29.6 (2.9)	14.8 (5.8)	-	-	86
364		13.2 (5.1)	19.8 (3.4)	-	-	67
680		-	19.8 (4.8)	-	-	95.7
α-Hydroxytropolone derivatives						
261		17.6 (3.3)	19.8 (2.9)	29.6 (2.0)	-	58
262		19.8 (2.9)	66.7 (0.9)	-	-	57.5
310		-	29.6 (2.5)	29.6 (2.5)	-	73

^{a}The therapeutic index, TI, (CC$_{50}$/MIC$_{80}$) is provided in parentheses to the right of the MIC$_{80}$ values. MIC$_{80}$ >71.4 μM.

Table 3. MIC$_{80}$ on *S. aureus* ATCC Strains and MRSA^a

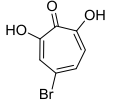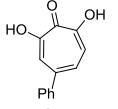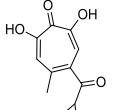| bacteria strains	antibiotics												compounds MIC$_{80}$ (μM)	
	FOX	OXA	GEN	CIP	ERY	CLI	LZD	DAP	VAN	TET	RIF	SXT	63	285
ATCC BAA 1026	POS	R	R	R	R	R	S	S	S	S	S	R	8.8	8.8
ATCC 25923	Neg	S	S	S	S	S	S	S	S	S	S	S	8.8	8.8
ATCC BAA 976	POS	R	S	S	R	S	S	S	S	S	S	S	13.2	8.8
ATCC BAA 977	Neg	S	S	S	R	R	S	S	S	S	S	S	8.8	8.8
ATCC 29213	Neg	S	S	S	S	S	S	S	S	S	S	S	8.8	8.8
Sa1	POS	R	S	R	R	R	S	S	S	S	S	R	8.8	8.8
Sa2	POS	R	S	R	R	S	S	S	S	S	S	S	8.8	8.8
Sa3	POS	R	S	R	R	R	S	S	S	S	S	S	8.8	8.8
Sa5	POS	R	S	R	R	R	S		S	S		S	8.8	8.8
Sa6	POS	R	S	R	R	R	S	S	S	S	S	S	8.8	8.8
Sa7	POS	R	S	S	R	S	S	S	S	S	S	S	8.8	8.8
Sa8	POS	R	S	R	R	S	S		S			S	8.8	8.8
Sa9	POS	R	S	R	R	R	S	S	S	S	S	S	8.8	8.8
Sa10	POS	R	S	R	R	R	S		S			S	8.8	8.8
Sa11	POS	R	S	R	R	R	S	S	S	S	S	S	8.8	8.8
Sa12	POS	R	S	R	R	R	S	S	S	S	S	S	8.8	8.8
Sa14	POS	R	S	R	R	R	S		S	S	S	S	8.8	8.8
Sa15	POS	R	S	R	R	R	S	S	S	S	R	R	8.8	5.9
Sa16	POS	R	S	R	R	R	S	S	S	S	S	S	8.8	8.8
Sa17	POS	R	S	R	R	S	S	S	S	S	S	S	8.8	8.8
Sa18	POS	R	S	R	R	S	S	S	S	S	S	S	8.8	8.8
Sa19	POS	R	S	S	R	R	S	S	S	S	S	S	8.8	8.8
Sa21	POS	R	S	S	R	S	S	S	S	S	S	S	8.8	5.9

^{a}R: resistance; S: sensitive; I: intermediate. Sa: MRSA. Cefoxitin (FOX); oxacillin (OXA); gentamicin (GEN); ciprofloxacin (CIP); erythromycin (ERY); clindamycin (CLI); linezolid (LZD); daptomycin (DAP); vancomycin (VAN); tetracycline (TET); rifampin (RIF); trimethoprim–sulfamethoxazole (SXT).

Table 4. MIC$_{80}$ on MDR Enterobacteriaceae for Compounds 284 and 363 and on MDR _A. baumannii_ for Compound 261[a]

bacteria strains	antibiotics											compounds MIC$_{80}$ (μM)		
	ESBL	SAM	TZP	CFZ	CRO	IPM	GEN	TOB	CIP	NIT	SXT	284	363	261
E. coli 35218	NEG	S	S	S	S	S	S	S	S	S	S	13.2	14.8	29.6
Ec1	POS	R	S	R	R	S	R	I	S	S	R	19.8	22.2	
Ec2	POS	I	S	R	R	S	R	I	R	S	R	13.2	9.8	
Ec3	POS	I	S	R	R	S	R	I	S	S	R	13.2	9.8	
Ec4	POS	R	S	R	R	S	S	R	R	S	S	19.8	14.8	
Ec5	POS	R	S	R	R	S	S	R	R	S	R	19.8	19.8	
Ab1		S		R	R	S	S	S	R	R	R			29.6
Ab2		S		R	R	I	R	S	R	R	R			19.8
Ab3		S	R	R	R	I	R	S	R	R	R			19.8
Ab4		S		R	R	I	R	S	R	R	R			19.8
Ab5		I		R	R	R	R	I	R	R	R			29.6

[a]R: resistance; S: sensitive; I: intermediate. Ec: _E. coli_; Ab: _Acinetobacter baumannii_. Extended spectrum beta-lactamases (ESBL); ampicillin–sulbactam (SAM); piperacillin–tazobactam (TZP); cefazolin (CFZ); ceftriaxone (CRO); imipenem (IPM); gentamicin (GEN); tobramycin (TOB); ciprofloxacin (CIP); nitrofurantoin (NIT); trimethoprim–sulfamethoxazole (SXT).

primary hits against _S. Saprophyticus_ and _S. aureus_ based on MIC$_{80}$ and CC$_{50}$ values, **63** and **285**, to determine if they inhibit other _S. aureus ATCC_ strains and methicillin-resistant _S. aureus_ (MRSA). The MRSA strains were collected at the St Louis VA Medical Center (STLVAMC) under STLVAMC Subcommittee on Research Safety (SRS)-approved protocols. All MRSA strains are cefoxitin-screen positive and resistant to oxacillin. As shown in Table 3, the MIC$_{80}$ of compounds **63** and **285** against _S. aureus_ ATCC strains and clinical MRSA strains ranged from 5.9 to 13.2 μM, similar to the value (8.8 μM) against the _S. aureus_ strain (ATCC 29213) used for the initial MIC$_{80}$ measurements. In addition to being resistant to oxacillin, 15 of 18 MRSA strains were also resistant to the fluoroquinolone class antibiotic ciprofloxacin, 12 were resistant to protein synthesis inhibitor class antibiotic clindamycin, one was resistant to the DNA-dependent RNA polymerase inhibitor class antibiotic rifampicin, and two were resistant to the folate synthesis inhibitor class antibiotic trimethoprim/sulfamethoxazole. Compounds **63** and **285** had similar potency in all drug-resistant _S. aureus_ strains, indicating that they have different target(s) from the existing antibiotics tested against which the strains had been profiled.

Inhibition of Multidrug-Resistant Gram-Negative Strains by Compounds 284, 363, and 261. Compounds **284** and **363** inhibited growth of _E. coli_. Therefore, we tested whether they could inhibit multidrug-resistant (MDR) _E. coli_. All tested MDR bacteria are resistant to at least three classes of antibiotics among quinolones/fluorquinolones, carbapenems, cephalosporins, aminoglycosides, and piperacillin–tazobactam. Compounds **284** and **363** inhibited five MDR _E. coli_ (Ec1-5) strains with MIC$_{80}$ values ≤30 μM (Table 4). We also tested **261**, an inhibitor of _A. baumannii_, for inhibition of five MDR _A. baumannii_ strains and found that it inhibited them with MIC$_{80}$ ≤30 μM (Table 4).

Time-Killing Curves and Bactericidal/Bacteriostatic Measurements. We next determined time-killing curves of representative inhibitors and whether they were bactericidal or bacteriostatic against _S. aureus_ (ATCC 29213) and _E. coli_ (ATCC 35218) strains. Compounds were diluted into CAMHB medium to a final concentration of 0, 1, 4, and 16 times their MIC$_{80}$s. Overnight cultures of the test bacteria were added to the compounds. Samples were taken immediately and approximately 3, 6, 24, and 30 h after the addition of compounds and plated onto blood agar. The numbers of

colonies appearing on the plate after 24 h of incubation at 37 °C were counted. Compounds **63** and **285** reduced the colony count by only 1 log$_{10}$ unit within 24 h in 1× MIC$_{80}$. However, at 4× MIC$_{80}$, they completely killed the bacteria after 5 or 8 h, while at 16× MIC$_{80}$, there was only a 2 log$_{10}$ reduction in the colony count within 24 h for both **63** and **285**. This paradoxical effect in which inhibition decreases over a range of increasing compound concentrations has been previously observed with β-thujaplicin (**47** in our nomenclature).[8] A similar paradoxical effect has also been described for β-lactam antibiotics against Gram positive bacteria[20−22] and for other antibiotics−microorganism combinations.[23,24] This phenomenon, which was demonstrated in vitro and in vivo,[24] is related to β-lactamase production,[24] alteration in the synthesis or activity of an autolysin,[25] binding to human albumin, as well as high-density inoculum of stationary cells.[26]

The time-killing curves for compounds **284** and **363** against _E. coli_ revealed a 1−2 log$_{10}$ reduction from 4 to 30 h for 1× and 4× MIC$_{80}$. At 16× MIC$_{80}$, there was about a 3 log$_{10}$ reduction within 6 h, then about a 2−4 log$_{10}$ reduction after 24 h for **284** and **363**. These results indicate that the troponoids can be bacteriostatic for _E. coli_ and bactericidal for _S. aureus_ under certain doses and compound exposures.

Compounds 63 and 285 Inhibit _S. aureus_ Independently of the Capsule and the CapF Protein. Nakano et al. reported that 3-isopropenyl-tropolone (**349**) can bind to CapF, which catalyzes synthesis of a key precursor of capsular polysaccharide.[27] Therefore, we asked if the MIC$_{80}$s of **63**, **285**, and **349** against _S. aureus_ strains G01 and F4 were altered relative to strain Newman. F4 is Newman with _ermB_-inactivated _cap5F_ gene and G01 is Newman with the _ermB_-inactivated _cap5G_ gene. Production of capsule polysaccharide is abolished in both the G01 and F4 strains. Compound **349** slightly inhibited the wild-type Newman but did not inhibit the F4 and G01 strains. However, both **63** and **285** inhibited Newman, F4 and G01 with similar MIC$_{80}$s (Table 5). Because the capsular protein is not essential for bacterial growth, from this growth inhibition assay, we cannot conclude whether the CapF is the target of the compounds tested or not, but the significant inhibition and/or killing of both wild type and capsular protein-ablated mutants indicate that neither capsule nor CapF protein are essential for action of **63** and **285**.

Table 5. MIC$_{80}$ of *S. aureus* Newman, G01, and F4 for Selected Compounds

comp#	MIC$_{80}$ (μM)		
	Newman[a]	F4[a]	G01[a]
63	13.2	13.2	13.2
285	13.2	13.2	13.2
349	66.7	100	100

[a]*S. aureus.*

DISCUSSION

In this study, we determined the antibacterial activities of 92 troponoids. Nine tropones (**51**, **62**, **63**, **282−285**, **348**, and **364**), three tropolones (**53**, **54**, and **338**), and two α-HTs (**261** and **262**) inhibited *S. aureus*/*S. saprophyticus* growth by ≥80% at <20 μM with CC$_{50}$s in human cells >50 μM. Compounds **261**, **284**, **310**, **363**, **364**, and **680** inhibited *E. coli*, and **261** and **310** inhibited *A. baumannii* growth by ≥80% at <30 μM with CC$_{50}$s >50 μM. Compounds **261**, **284**, **363**, and **364**, which inhibited Gram-negative bacteria, also inhibited Gram-positive bacteria modestly, but the opposite is not true, as **51**, **53**, **282**, **283**, **285**, **338**, and **348** inhibited *S. aureus* at <20 μM, but could not inhibit *E. coli*, *A. baumannii*, or *P. aeruginosa*. The broad anti-bacterial activity of β-thujaplicin (Hinokitiol, **47**) and γ-thujaplicin (**48**) was reported several decades ago.[8,28,29] Our results revealed modest inhibition of both Gram-positive bacteria (*S. aureus*) and Gram-negative rods (*E. coli* and *A. banmannii*), which is consistent with previous reports (Table S1). Two α-HT, **261** and **262**, also showed broad inhibition against both Gram-positive and Gram-negative bacteria (Table 2). However, the CC$_{50}$ values were around 50 μM, so there is little to no TI compared to their effects on human cells.

Nine of 15 benzoylated tropolones (**51**, **62**, **63**, **282**, **283**, **284**, **285**, **348**, and **364**) inhibited growth of *S. aureus* by ≥80% at <20 μM with CC$_{50}$ values >50 μM (Table 2). Two benzoylated tropolones (**281** and **339**) had modest inhibition. These benzoylated tropolones all have a troponoid ring connected to a benzoate through an ester linkage, or in the case of **284**, **348**, and **364**, a thioester linkage. Because the addition of the benzene ring did not affect inhibition of bacterial growth significantly, we assume that the benzene ring is not a primary determinant of antibacterial activity, but they can affect the interaction between the tropolone ring and the target.

Compounds **284**, **364**, and **363** inhibited MDR *E. coli* with MIC$_{80}$ ≤30 μM. **364** is a derivative of **284** lacking the methyl group on the benzene moiety, and it had an efficacy similar to **284**. As the thioester bond in **284** is unlikely to be stable in culture, we synthesized one of the putative esterase products, thiotropolone (compound **363**). As shown in Table 2, **363** was more active than **284** (14.8 μM vs 24.1 μM). This indicates that the minimal active component of our primary screening hit was thiotropolone **363**. To expand the assessment of thiotropolones as inhibitors of *E. coli* growth, Dr. Elgendy synthesized 10 new compounds with different modifications on the troponoid ring and the right arm (**677−686**). Compounds **677**, **678**, **680**, **681**, **684**, and **685** have the thiotropolone core structure and they inhibited *E. coli* growth at <20 μM (Table S1 and Figure S1). As expected, compound **683**, which has the oxygen replaced sulfur atom next to the ketone group on the troponoid ring, is inactive against *E. coli*. Compound **686** has

the thiotropolone core, but is inactive, indicating that the right arm somehow participates in the interaction of thiotropolone with the bacterial target, potentiates compound degradation, and/or induces their efflux from the cells. These data further demonstrate that thiotropolone is the core structure in these compounds for the anti-bacterial activity against *E. coli*, but that modifications to the tropolone ring and thiol moieties can affect efficacy. **284** and **363** can also inhibit growth of the fungal pathogen *Cryptococcus neoformans* with an MIC$_{80}$ of 0.25 μM[15] and unpublished data. However, it is unknown whether they inhibit the bacteria and *C. neoformans* by the same mechanisms.

Two compounds, **63** and **285**, also inhibited other *S. aureus* ATCC strains and MRSA *S. aureus* strains collected from patients with similar MIC$_{80}$ values as against ATCC reference strains. These clinical isolates and ATCC strains had extensive but differing resistance patterns to a set of common clinically relevant antibiotics including oxacillin, gentamicin, ciprofloxacin, erythromycin, clindamycin, and trimethoprim−sulfamethoxazole in addition to methicillin. For Gram-negative rods, compounds **284**, **363**, and **364** inhibited *E. coli* and MDR-*E. coli* with MIC$_{80}$ ≤30 μM. Meanwhile, compound **261** inhibited MDR *A. baumannii* with MIC$_{80}$ ≤30 μM. These clinical isolates also had extensive but differing resistance patterns to a set of common clinically relevant antibiotics including ampicillin−sulbactam, piperacillin−tazobactam, cefazolin, ceftriaxone, cefepime, gentamicin, tobramycin, ciprofloxacin, nitrofurantoin, and trimethoprim−sulfamethoxazole. The inhibition of these MDR bacteria indicates the compounds tested, and possibly the other troponoids, have different bacterial targets from the common existing antibiotics.

The biological effects of troponoid compounds are typically due to coordination of cations in the active sites of metalloenzymes.[30−32] For example, the α-HTs inhibit the HIV ribonuclease H by coordinating the two Mg^{2+} ions in the active site,[33] and they are believed to act the same way against the hepatitis B virus ribonuclease H.[34] Similarly, tropolone has been reported to inhibit several Zn^{2+}-dependent metalloenzymes.[35−37] Finally, CapF is a bifunctional metalloenzyme which is essential in the biosynthetic route of capsular polysaccharide. Isothermal titration calorimetry demonstrates that 3-isopropenyl-tropolone (**349**) binds (K_d = 27 ± 7 μM) to the cupin domain of CapF. In addition, the crystal structure of the enzyme−inhibitor complex shows that the compound engages the essential Zn^{2+} ion necessary for the first reaction catalyzed by the enzyme and alters the coordination sphere of the metal, leading to the overall destabilization of the enzyme.[27] However, from the MIC$_{80}$ against *S. aureus* Newman and F4 (with *ermB*-inactivated *capSF* gene), **349** can only slightly inhibit Newman growth and cannot inhibit the F4 growth, while **63** and **285** can significantly inhibit the growth of Newman and F4. In addition, the time-killing assay showed that **63** and **285** are bactericidal for a MRSA strain. Although this growth inhibition assay does not distinguish whether **63** and **285** can bind to CapF or not, we can conclude that the target(s) of **63** and **285** must be something in addition to or other than CapF because the capsule is not essential for the growth of *S. aureus*. Through screening of a chelator fragment library, tropolone was identified as an inhibitor of the Zn^{2+}-dependent virulence factor, *P. aeruginosa* elastase (LasB).[37] However, none of the troponoid compounds inhibited growth of *P. aeruginosa* at <71.4 μM in our assay. This could be because LasB is not essential for the growth of *P. aeruginosa*,
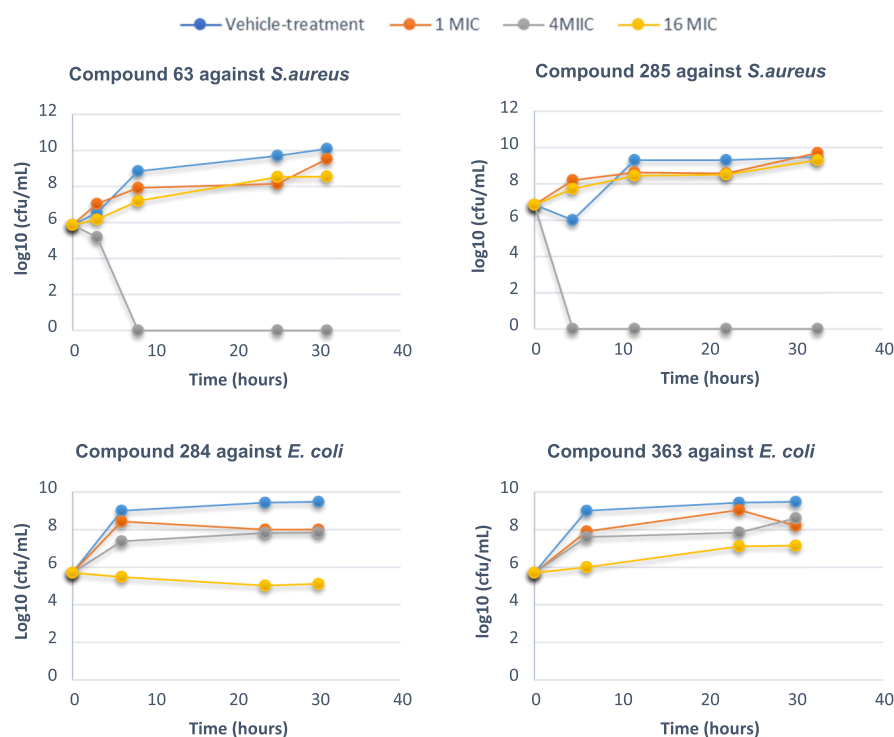
Figure 2. Time-killing curves for compounds **63** and **285** against *S. aureus* (ATCC 29213) and for **284** and **363** against *E. coli* (ATCC 35218).

although our growth inhibition assay cannot determine whether the troponoids bind to LasB or not. The mechanism(s) of troponoid inhibition is unknown, but the bactericidal property of investigated compounds (**63** and **285**, Figure 2) in certain concentrations indicates that they disrupt the function of bacterial target(s) essential for bacterial viability.

These data greatly expand knowledge regarding the antibacterial efficacy of the troponoids. Importantly, the activity of these compounds against a panel of highly drug-resistant Gram-positive and Gram-negative bacteria indicates that they act by mechanism(s) distinct from existing clinically used antibiotics. Therefore, this study opens up a new avenue for development of novel troponoids antibiotics to address the critical and urgent need for novel drugs to combat serious bacterial infections.

EXPERIMENTAL SECTION

Compound Acquisition and Synthesis. The compounds employed are listed in Table S1.

Compounds were acquired commercially or were synthesized as described below. Compounds **46−57** and **195** were acquired from the National Cancer Institute (NCI) Developmental Therapeutics Program. Compounds **60−63**, **210**, **281−285**, and **348−350** were purchased. Compound **172** was synthesized according to a published procedure.[38] Compounds **106−120**, **143−147**, **173**, **273−274**, and **335** were synthetized from kojic acid as previously described.[11,39−41] Compounds **257−259**, **280**, **308−313**, **315**, **317−319**, **336**, and **347** were synthesized as previously described.[15,42] Compounds **261−264** were made using the Banwell method.[43] Compound **363** was synthesized from 2-cholorotropone and sodium hydrosulfide.[44] Compound **364** was synthesized from **363** according to the procedure of Nozoe.[45] Compound **365** was synthesized according to a published procedure.[46] For the synthesis of

compounds **675−682**, **684−686** (**675** = **363** and **676** = **364**), see Supporting Information Compounds were ≥95% pure by ^{1}H NMR analysis. The analytical data for all published compounds are consistent with that reported previously. They were dissolved in DMSO at 10 mM and stored in opaque tubes at −80 °C.

Bacterial Strains. The commercially acquired bacterial strains were obtained from the American Type Culture Collection (ATCC). The clinical MRSA, MDR Enterobacteriaceae, and *A. baumannii* strains were collected from the microbiology laboratory at the John Cochran division of the St. Louis VA Health care system (STLVAHCS) under STLVAHCS Subcommittee on Research Safety (SRS)-approved protocols. *S. aureus* Newman, G01, and F4 were kindly provided by Dr. Jean Lee. G01 is Newman with *ermB*-inactivated *cap5G* gene and F4 is Newman with *ermB*-inactivated *cap5F* gene.[47]

Determination of the Minimum Inhibitory Concentration. MIC_{80}s were determined by the broth microdilution method recommended by the Clinical and Laboratory Standards Institute (CLSI) in CAMHB. In the preliminary screening, three compound concentrations were used: 5.8, 20.4, and 71.4 μM; In quantitative MIC_{80} measurements, a 1.5-fold dilution series of the compounds was prepared in CAMHB. Overnight bacterial culture was added to the diluted compounds in a 96-well plate after adjusting the bacterial concentration to achieve a 5×10^5 CFU/mL final concentration. After 16−24 h incubation at 35 ± 2 °C, the plates were read at 630 nM in a microplate reader. The MIC_{80} was defined as the concentration of an antibacterial agent that inhibited bacteria growth ≥80% compared to untreated control cultures. All values were determined at least twice independently, and the average number is reported.

MTS Cytotoxicity Assays (CC_{50}). HepDES19 cells[19] (1.0 × 10^4 cells per well) were seeded in 96-well plates and

incubated in Dulbecco's modified eagle medium with 10% fetal bovine serum plus 1% penicillin/streptomycin solution, 1% nonessential amino acids, and 1% glutamine. The compounds were diluted in the medium to the indicated concentrations to a final concentration of 1% DMSO and added to the cells 48 h after plating, with each concentration tested in triplicate. Soluble MTS reagent [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, Promega] was added 72 h after incubation, the cultures were incubated for 90 min, and absorbance was read at 490 nm. The CC_{50} was calculated as the concentration of the inhibitor required to reduce cell viability 50% relative to untreated cells. The data are plotted as log[inhibitor] versus response and fit to a variable slope model using Graph Pad Prism.[15]

Time-Killing Curve and Bactericidal/Bacteriostatic Measurement. Compounds were diluted into CAMHB medium containing 0 (vehicle-treatment control), 1, 4, or 16 times the MIC_{80}. Approximately 10^5 CFU/mL of the test bacteria from overnight cultures were added to the compound solutions. Samples were taken immediately and 3, 6, 24, and 30 h after the addition of compounds and plated onto blood agar. The numbers of colonies appearing on the plate after 24 h of incubation at 37 °C were counted.[48]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b01754.

MIC$_{80}$ and CC$_{50}$ results and structures for all 92 tested troponoids and synthesis and characterization of compounds 675−682, 684−686 (PDF)

■ AUTHOR INFORMATION

Corresponding Author
*E-mail: feng.cao@va.gov. Phone: +1 (314) 289-6358. Fax: +1(314) 289-7920 (F.C.).

ORCID Ⓞ
Feng Cao: 0000-0002-4820-6628
Ryan P. Murelli: 0000-0002-4247-3936
Bahaa Elgendy: 0000-0003-4800-7976
John E. Tavis: 0000-0002-8711-4240

Notes
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