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Synthesis and Evaluation of Troponoids as a New Class of Antibiotics

Feng Cao,* Cari Orth,† Maureen J. Donlin,* Patrick Adegboyea,† Marvin J. Meyers,§ Ryan P. Murelli,¶ Mohamed Elagawany,*† Bahaa Elgendy,*† and John E. Tavis*oa

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. 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. 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 2,4,6-cycloheptatrien-1-one (AH-T) character. Tropon (2,4,6-cycloheptatrien-1-one) has an alcohol (or an enol group) next to the ketone. Tropolone (2-hydroxytropolone) is the alcohol form of AH-T. AH-T is also present in the labda diterpenoids, which have been shown to have significant antimicrobial activity. 2,3 Troponoid compounds generally have higher antibacterial activity against human pathogens than compounds with similar MIC80 values. 2,3

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 (MIC50) and cytotoxicity (CC50) against human hepatoma cells. Sixteen troponoids inhibited S. aureus/E. coli/A. baumannii growth by ≥80% growth at ≤30 μM with CC50 values >50 μM. Two selected tropones (63 and 285) inhibited 18 methicillin-resistant S. aureus (MRSA) strains with similar MIC50 values as against a reference strain. Two selected thiotropolones (284 and 363) inhibited multidrug-resistant (MDR) E. coli with MIC50 ≤30 μM. One α-HT (261) inhibited MDR A. baumannii with MIC50 ≤30 μM. This study opens new avenues for development of novel troponoid antibiotics to address the critical need to combat MDR bacterial infections.
Hydroxytropolone (α-HT) has an additional alcohol group on C7 of the troponoid ring (Figure 1).

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

Troponoid derivatives can have antibacterial, antiviral, anti-inflammatory, antioxidative, and insecticidal properties.

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 were tropones, 26 were tropolones, and 48 were 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 (ATCC 19600), 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 × 10^3 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 Table S1.

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

<table>
<thead>
<tr>
<th>organism</th>
<th>compounds concentration (μM)</th>
<th>5.8</th>
<th>20.4</th>
<th>71.4</th>
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<td>S. saprophyticus</td>
<td>percentage of compounds (%)</td>
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<td>9.8</td>
<td>39.1</td>
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<td>percentage of compounds (%)</td>
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<td>8.7</td>
<td>20.7</td>
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<tr>
<td>A. baumannii</td>
<td>percentage of compounds (%)</td>
<td>0</td>
<td>0</td>
<td>15.2</td>
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<td>P. aeruginosa</td>
<td>percentage of compounds (%)</td>
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</table>

MIC_{50} 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 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_{50}), 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 troponoids that inhibited S. aureus, 53, 54, and 338 inhibited growth by >80% at <20 μM with CC_{50} >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 troponoids, 47 to 50, 52, 55, 195, 340 to 345, and 349 had moderate substitutions on the troponoid ring and had decreased or no activity. The 26 tropones tested were all variants of 53 with modifications to the troponol 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 baumannii 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} >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$_{50}$ and TI Values of *E. coli*, *S. aureus*, *A. baumannii*, and *P. aeruginosa* and CC$_{50}$ for Selected Compounds

<table>
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<th>Compound Number</th>
<th>Compound Structure</th>
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<th>S. aureus (ATCC29213)</th>
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<th>P. aeruginosa (ATCC27853)</th>
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<td>-</td>
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Table 3. MIC<sub>80</sub> on S. aureus ATCC Strains and MRSA<sup>a</sup>

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<sup>a</sup>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).
primary hits against \textit{S. Saprophyticus} and \textit{S. aureus} based on MIC$_{90}$ and CC$_{50}$ values, 63 and 285, to determine if they inhibit other \textit{S. aureus} ATCC strains and methicillin-resistant \textit{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$_{90}$ of compounds 63 and 285 against \textit{S. aureus} ATCC strains and clinical MRSA strains ranged from 5.9 to 13.2 $\mu$M, similar to the value (8.8 $\mu$M) against the \textit{S. aureus} strain (ATCC 29213) used for the initial MIC$_{90}$ 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 \textit{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 \textit{E. coli}. Therefore, we tested whether they could inhibit multidrug-resistant (MDR) \textit{E. coli}. All tested MDR bacteria are resistant to at least three classes of antibiotics among quinolones/fluoroquinolones, carbapenems, cephalosporins, aminoglycosides, and piparacillin–tazobactam. Compounds 284 and 363 inhibited five MDR \textit{E. coli} (Ec1-S) strains with MIC$_{90}$ values $\leq$30 $\mu$M (Table 4). We also tested 261, an inhibitor of \textit{A. baumannii}, for inhibition of five MDR \textit{A. baumannii} strains and found that it inhibited them with MIC$_{90}$ $\leq$30 $\mu$M (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 \textit{S. aureus} (ATCC 29213) and \textit{E. coli} (ATCC 35218) strains. Compounds were diluted into CAMHB medium to a final concentration of 0, 1, 4, and 16 times their MIC$_{90}$. 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 $^\circ$C were counted. Compounds 63 and 285 reduced the colony count by only 1 log$_{10}$ unit within 24 h in 1X MIC$_{80}$. However, at 4X MIC$_{80}$, they completely killed the bacteria after 5 or 8 h, while at 16X 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 $\beta$-thujaplicin (47 in our nomenclature). A similar paradoxical effect has also been described for $\beta$-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,$^{25}$ is related to $\beta$-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 \textit{E. coli} revealed a 1–2 log$_{10}$ reduction from 4 to 30 h for 1X and 4X MIC$_{80}$. At 16X 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 \textit{E. coli} and bactericidal for \textit{S. aureus} under certain doses and compound exposures.

**Compounds 63 and 285 Inhibit \textit{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$_{90}$ of 63, 285, and 349 against \textit{S. aureus} strains G01 and F4 were altered relative to strain Newman. F4 is Newman with ermB inactivated \textit{capSF} gene and G01 is Newman with the \textit{ermB}-inactivated \textit{capSG} 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$_{90}$ (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. MIC80 of *S. aureus* Newman, G01, and F4 for Selected Compounds

<table>
<thead>
<tr>
<th>comp#</th>
<th>MIC80 (μM)</th>
<th>Newman*</th>
<th>F4*</th>
<th>G01*</th>
</tr>
</thead>
<tbody>
<tr>
<td>63</td>
<td>13.2</td>
<td>13.2</td>
<td>13.2</td>
<td></td>
</tr>
<tr>
<td>285</td>
<td>13.2</td>
<td>13.2</td>
<td>13.2</td>
<td></td>
</tr>
<tr>
<td>349</td>
<td>66.7</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

*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 CC50s in human cells >50 μM. Compounds 261, 284, 310, 363, 364, and 680 inhibited *E. coli* and *A. baumannii* growth by ≥80% at <30 μM with CC50s >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.28,29 Our results revealed modest inhibition of both Gram-positive bacteria (*S. aureus*) and Gram-negative rods (*E. coli* and *A. baumannii*), 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 CC50 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 CC50 values >50 μM (Table 2). Two benzoylated tropolones (281 and 339) had modest inhibition. These benzoylated tropolones all have a tropolone ring connected to a benzooate 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 MIC80 ≤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. Eldygen synthesized 10 new compounds with different modifications on the tropolone 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 tropolone 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 MIC80 of 0.25 μM 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 MIC80 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 MIC80 ≤30 μM. Meanwhile, compound 261 inhibited MDR *A. baumannii* with MIC80 ≤30 μM. These clinical isolates also have extensive but differing resistance patterns to a set of common clinically relevant antibiotics including ampicillin–sulbactam, piperacillin–tazobactam, ceftazolin, 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 Mg2+ ions in the active site,51 and they are believed to act the same way against the hepatitis B virus ribonuclease H.33 Similarly, tropolone has been reported to inhibit several Zn2+-dependent metalloenzymes.33–35 Finally, CapF is a bifunctional metalloenzyme which is essential in the biosynthetic route of capsular polysaccharide. Isothermal titration calorimetry demonstrates that 3-isoprophenyl-tropolone (349) binds (KD = 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 Zn2+ 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.36 However, from the MIC80 against *S. aureus* Newman and F4 (with ermB-inactivated cap5F 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 Zn2+-dependent virulence factor, *P. aeruginosa* elastase (LasB).37 However, none of the troponoid compounds inhibited growth of *P. aeruginosa* at <7.14 μM in our assay. This could be because LasB is not essential for the growth of *P. aeruginosa*. 

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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 hydrosulfitde.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 1H 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<sub>50</sub>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<sub>50</sub> 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<sup>5</sup> 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<sub>50</sub> 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<sub>50</sub>).** HepDES19 cells (1.0 × 10<sup>4</sup> 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% glucose. 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 CC50 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.\(^{16}\)

**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 CC50 results and structures for all 92 tested troponoids and synthesis and characterization of compounds 675–682, 684–686 (PDF)

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**Notes**

The authors declare no competing financial interest. The contents do not represent the views of the U.S. Department of Veterans Affairs.

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