



ARTICLE

# Synthesis and antibiotic activity of novel acylated phloroglucinol compounds against methicillin-resistant *Staphylococcus aureus*

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## Abstract

The rise in antibiotic resistance among pathogenic microorganisms has created an imbalance in the drugs available for treatment, in part due to the slow development of new antibiotics. Cystic fibrosis (CF) patients are highly susceptible to antibiotic-resistant pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA). Phloroglucinols and related polyketide natural products have demonstrated antimicrobial activity against a number of Gram-positive bacteria including *S. aureus*. In this study, we investigated a series of acylated phloroglucinol derivatives to determine their potential as lead compounds for the design of novel therapeutics. To assess the activity of these compounds, we determined the minimum inhibitory and bactericidal concentration (MIC and MBC, respectively), the minimum biofilm inhibitory and biofilm eradication concentration (MBIC and MBEC, respectively), and evaluated hemolytic activity, as well as their interaction with clinically relevant antibiotics. Of the 12 compounds tested against MRSA and methicillin-susceptible strains, four showed MIC values ranging from 0.125 to 8 µg ml<sup>-1</sup> and all of them were bactericidal. However, none of the compounds were able to eradicate biofilms at the concentrations tested. Three of the four did not display hemolytic activity under the conditions tested. Further studies on the interactions of these compounds with clinically relevant antibiotics showed that phlorodipropanophenone displayed synergistic activity when paired with doxycycline. Our results suggest that these acylated phloroglucinols have potential for being further investigated as antibacterial leads.

## Introduction

The emergence of multidrug resistant bacterial infections is a major health threat [1]. Agencies like the World Health Organization and the Public Health Agency of Canada [2] have highlighted the need for investment in research and development of new antibiotics. A remarkable example is

the Gram-positive bacterium *Staphylococcus aureus*, a ubiquitous commensal of the human skin. Naturally susceptible to most antibiotics, *S. aureus* is capable of causing infections in predisposed individuals and can acquire formidable antibiotic resistance [3]. One of the most notorious antibiotic-resistant members of the species is methicillin-resistant *S. aureus* (MRSA) [4], which are grouped into two categories referred as hospital-associated (HA-MRSA) and community-associated (CA-MRSA) [5]. MRSA is also a concerning pathogen for people with the genetic disease cystic fibrosis (CF) [6]. Studies show that lower airway inflammation in children with CF is associated with early colonization with *S. aureus* [7] and that the prevalence of MRSA in CF patients is high [8, 9].

Microorganisms such as bacteria and fungi produce chemical compounds that have been successfully used as therapeutic antibiotics [10]. Lichenized fungi are a rich source of mostly under-explored natural products, many of which have chemical structures that suggest an origin from acetate via the polyketide pathway. One of the most widely occurring lichen natural products is the dibenzofuran usnic acid (UA), and the biological activity of this polyketide has

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been extensively studied. The biosynthesis of UA has been demonstrated to proceed via the intermediate methylphloracetophenone (MPA [11], Fig. 1) which is produced from acetyl-CoA, malonyl-CoA, and *S*-adenosylmethionine (SAM) by a polyketide synthase (PKS). The intermediate MPA is in turn dimerized by an oxidative  $P_{450}$ -type enzyme to produce UA. The biological activity of MPA vs. UA has recently been examined [12] and it was demonstrated that UA is more bioactive than MPA in a series of antibacterial and biofilm disruption assays. However, a systematic examination of the biological activity of other related phloracetophenone analogs has not yet been reported. We chose to explore this chemical space as a potential source of novel bioactivity. Although MPA is a known natural product none of the compounds reported here appear to be of natural origin.

In this study, we synthesized 12 novel phloracetophenone analogs (Compounds 1–12, Schemes 1–3, Fig. 2) and explored their biological activity against *S. aureus* ATCC 29213 and MRSA strains isolated from CF patients. Our results show that 4 of the 12 novel compounds displayed promising activity as a monotherapy and one strongly synergized with doxycycline. These results suggest that phloracetophenone analogs may serve as promising lead compounds for the design of novel antibiotics.

## Results and discussion

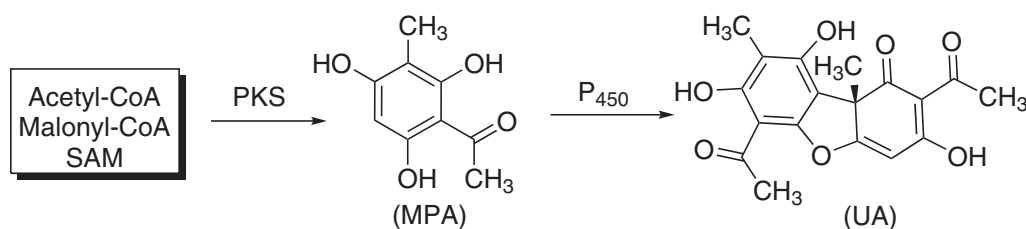
### Synthesis of phloroglucinol compounds

We first set out to synthesize a series of analogs of MPA where the methyl ketone had been replaced with a

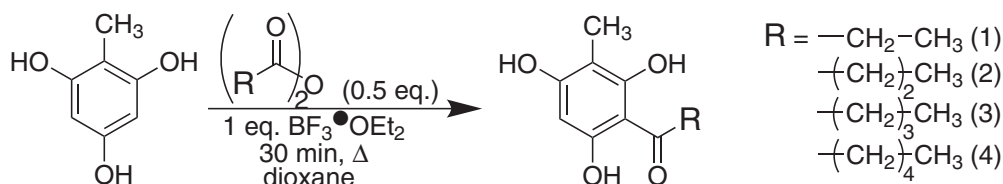
sequentially longer hydrocarbon chain of 3–6 carbon atoms in length. The synthesis of compounds 1–4 is summarized in Scheme 1. Commercially available trihydroxytoluene was reacted with 0.5 equivalent of the appropriate anhydride in the presence of 1 equivalent of Lewis acid ( $\text{BF}_3 \cdot \text{OEt}_2$ ) using dioxane as the solvent. Using the anhydride as the limiting reagent resulted in a correspondingly low yield (18–40%), but prevented over acylation of the substrate. We also undertook the synthesis of a series of acylated phloroglucinols, compounds 5–8, as described in Scheme 2. Phloroglucinol was dissolved in carbon disulfide and nitrobenzene and treated with 10 equivalents of the appropriate acid chloride using  $\text{AlCl}_3$  (10 equivalents) as catalyst. Heating for one hour followed by work-up afforded compounds 5–8 in a range of 41–53% yield. A series of diacylated phloroglucinol derivatives were synthesized as described in Scheme 3. Phloroglucinol was dissolved in dioxane and reacted with an excess of the appropriate anhydride (10 equivalents) and an excess (10 equivalents) of Lewis acid. Heating for 30 min followed by work up produced diacylphloroglucinols 9–12 in a range of 17 to 55% yield. The complete characterization data for each compound is provided in the supplementary material

### Evaluation of antibiotic properties

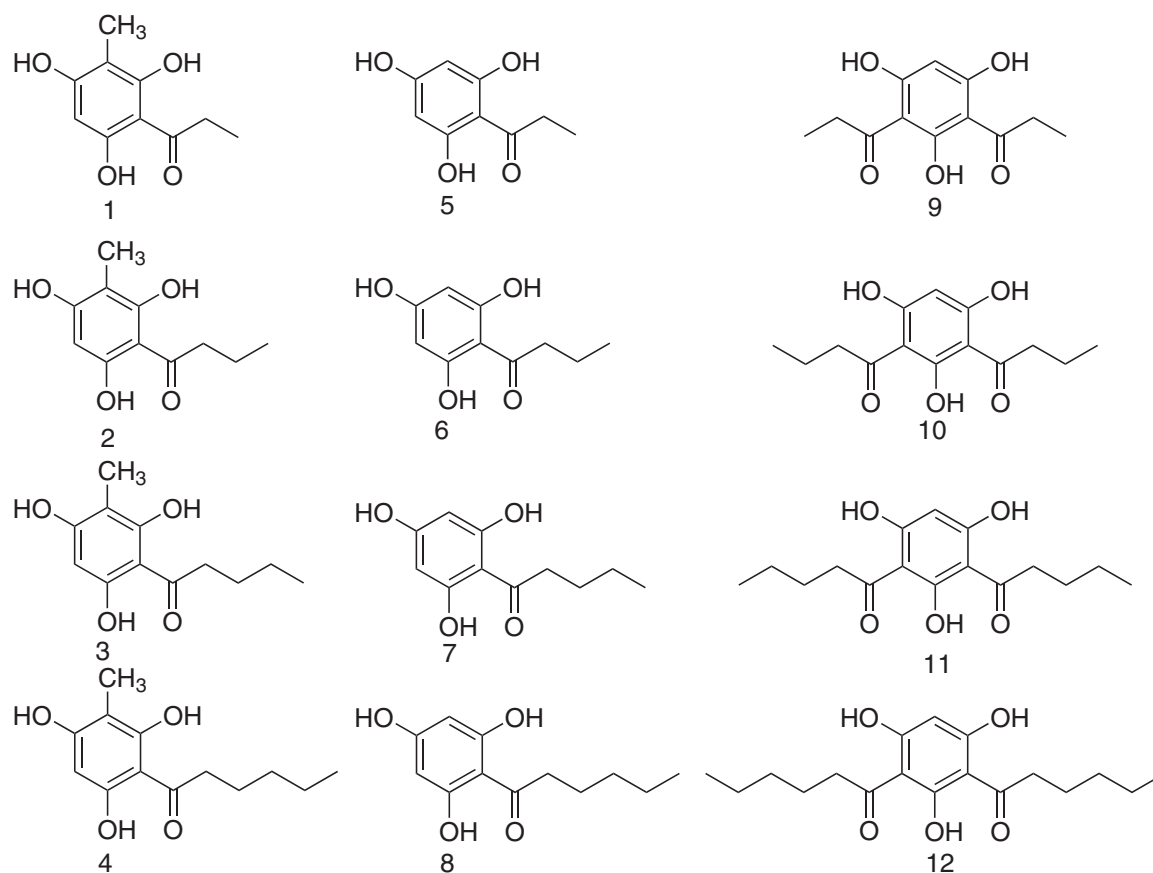
The minimum inhibitory concentration (MIC) of UA against *S. aureus* ranges between 6 and  $32 \mu\text{g ml}^{-1}$  depending on the strain [12]. To evaluate the antibiotic activity of the synthesized compounds, we performed MIC assays using a laboratory strain of *S. aureus* (ATCC 29213) and MRSA strains isolated from the sputum of CF patients [13]. Table 1 shows MICs of the synthesized compounds. Group 3 had the



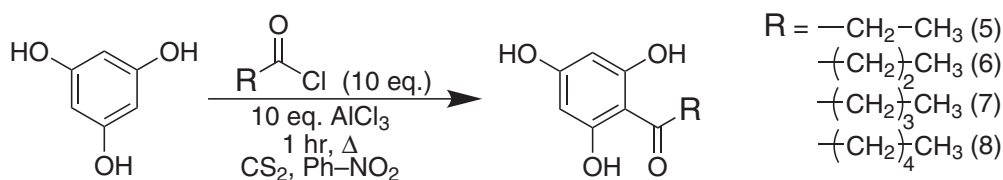
**Fig. 1** The biosynthesis of usnic acid (UA) from acetyl-CoA, malonyl-CoA, and *S*-adenosylmethionine (SAM) has been demonstrated to proceed via methylphloracetophenone (MPA)



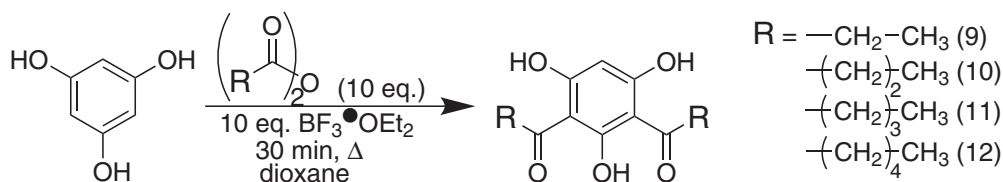
**Scheme 1** The synthesis of compounds 1–4 is shown



**Fig. 2** Chemical structure of the synthesized compounds. Compounds were grouped according to the synthesis scheme. Group 3 contains the most bioactive compounds



**Scheme 2** The synthesis of compounds 5–8 is shown



**Scheme 3** The synthesis of compounds 9–12 is shown

lowest MICs, ranging between 0.125 and 8  $\mu\text{g ml}^{-1}$ . Among them, compound **11** had the lowest MIC against all the strains of *S. aureus* tested ranging from 0.125 to 0.5  $\mu\text{g ml}^{-1}$ , while compounds **9** and **10** had MIC values ranging from 2 to 8  $\mu\text{g ml}^{-1}$ . The common structural feature for the four most active compounds (**9–12**) is a diketo moiety with a

mutual *ortho* phenolic group. The compounds differ only in the chain length of the alkyl group on the anhydride that was used in the acylation step. We then tested the ability of compounds **9–12** to effectively kill *S. aureus* by performing minimum bactericidal concentration (MBC) assays. All compounds displayed a MBC to MIC ratio lower than 4 for

**Table 1** Minimum inhibitory concentration (MIC) of the 12 novel phloroglucinol derivatives against *S. aureus* strains

Compound no.	MIC ( $\mu\text{g ml}^{-1}$ )							
	CF66 (MSSA)	ATTC 29213	CF225 (MRSA)	CF188 (MRSA)	CF224 (MRSA)	CF4 (MRSA)	CF250 (SCV MRSA)	CF3 (MRSA)
1	>32	>32	>32	>32	>32	>32	>32	>32
2	>32	>32	>32	>32	>32	>32	>32	>32
3	>32	>32	>32	>32	>32	>32	>32	>32
4	>32	>32	>32	>32	>32	>32	>32	>32
5	>32	>32	>32	>32	>32	>32	>32	>32
6	>32	>32	>32	>32	>32	>32	>32	>32
7	>32	>32	>32	>32	>32	>32	>32	>32
8	>32	>32	>32	>32	>32	>32	>32	>32
9	8	2	2	2	2	4	4	8
10	8	2	4	8	8	8	4	8
11	0.25	0.25	0.5	0.125	0.125	0.25	0.5	0.125
12	0.5	0.25	0.5	0.5	0.25	0.5	1	0.5

Values presented are the median of three biological replicates

SCV small colony variant

**Table 2** MBC and ratio of MBC to MIC for the four novel phloroglucinol derivatives against *S. aureus* strains

Compound no.	<i>S. aureus</i> CF3		<i>S. aureus</i> CF4		<i>S. aureus</i> CF66		<i>S. aureus</i> CF188	
	MBC ( $\mu\text{g ml}^{-1}$ )	MBC/MIC ratio	MBC ( $\mu\text{g ml}^{-1}$ )	MBC/MIC ratio	MBC ( $\mu\text{g ml}^{-1}$ )	MBC/MIC ratio	MBC ( $\mu\text{g ml}^{-1}$ )	MBC/MIC ratio
9	16	2	16	4	16	2	8	4
10	32	4	32	4	32	4	32	4
11	0.5	4	0.5	2	0.5	2	1	8
12	1	2	1	2	1	2	1	2

all the strains tested with exception of compound **11** and strain CF188 (Table 2), indicating a bactericidal action [14]. We also evaluated the ability of the synthesized phloroglucinol compounds to eradicate biofilms by performing the minimum biofilm eradication concentration (MBEC) assay (Suppl. Table 1). None of the four compounds from group 3 that had antibacterial activity possessed biofilm eradication activity (Suppl. Table 2).

To further investigate the therapeutic potential of the four active compounds, we evaluated their hemolytic properties against red blood cells (RBC) (Table 3). Only **11** displayed minor hemolytic activity of 8% at the highest concentration tested ( $32 \mu\text{g ml}^{-1}$ ), suggesting it would be unsuitable for therapeutic purposes. Furthermore, the lack of hemolytic activity for compounds **9**, **10**, and **12** suggests that this class of compounds may have promising selectivity against bacterial cells. Hence, more research on understanding the mechanism of action and the apparent selectivity is merited.

We also investigated the interaction of the compounds with clinically relevant antibiotics that have various mechanisms of action. We selected compound **9** as representative for checkerboard assays as it displayed strong

**Table 3** In vitro hemolysis of ovine red blood cells by novel phloroglucinol derivatives

Compound no.	Percentage hemolysis <sup>a</sup>		
	$32 \mu\text{g ml}^{-1}$	$16 \mu\text{g ml}^{-1}$	$8 \mu\text{g ml}^{-1}$
9	$0.60 \pm 1.14$	$1.07 \pm 1.48$	$0.74 \pm 1.18$
10	$0.17 \pm 1.08$	$0.07 \pm 0.56$	$0.28 \pm 1.0$
11	$8.10 \pm 0.89$	$0.49 \pm 0.55$	$-0.52 \pm 0.93$
12	$2.86 \pm 1.88$	$2.27 \pm 2.14$	$1.44 \pm 1.49$

<sup>a</sup>Values presented are the mean of three biological replicates  $\pm$  SD

activity, but without hemolysis. As shown in Table 4, Compound **9** interacted additively with oxacillin, cephalexin, rifampicin, and trimethoprim, and acted synergistically with doxycycline. This trend was seen in ATCC 29213 and the MRSA strains. Studies have shown that synergies often occur between drugs that target the same cellular process [15] and also can happen due to modulation of intracellular drug concentrations. Doxycycline targets the small ribosome subunit (30S subunit) interrupting protein synthesis. In a study conducted by Sahuquillo-Arce et al. it

**Table 4** Interaction of Compound **9** with several clinically relevant antibiotics against *S. aureus*

Antibiotic	<i>S. aureus</i> ATCC 29213		<i>S. aureus</i> CF225	
	FIC Index	Interpretation	FIC Index	Interpretation
Oxacillin	0.625	Additive	0.75	Additive
Cephalexin	1	Additive	1	Additive
Rifampicin	0.75	Additive	1	Additive
Doxycycline	0.325	Synergistic	0.325	Synergistic
Trimethoprim	1.5	Additive	1.5	Additive

Values presented are the median of three biological replicates

was shown that linezolid interacted synergistically with doxycycline [16]. Linezolid binds to the 50S ribosomal subunit inhibiting protein synthesis. Therefore, the synergistic interaction between doxycycline and linezolid occurs because the two compounds act on interacting targets [16]. It is tantalizing to speculate that the mechanism of action of compound **9** is related to the inhibition of protein synthesis, a hypothesis worth to further be tested.

## Conclusion

In the present study, we have synthesized 12 phloroglucinol derivatives and characterized their antibiotic properties against *S. aureus*, an important CF pathogen. Diacylated derivatives displayed the strongest bactericidal activity against MRSA clinical isolates. Furthermore, these compounds displayed apparent selectivity in their action, as they were not hemolytic. Of interest, compound **9** interacted synergistically with doxycycline. Hence, it could potentially be used as a drug combination to treat highly resistant MRSA strains.

## Experimental procedures

### Bacterial strains and growth conditions

Strains (Suppl. Table 1) were grown in Tryptic soy broth (TSB) (Difco) at 37 °C and 230 rpm shaking for routine overnight culturing unless otherwise stated.

### MIC and MBC assays

The CLSI protocol for determining the MIC and MBC of antibiotic activities in *S. aureus* was followed [17]. Briefly, an overnight culture was diluted equivalent to a 0.5 McFarland standard and then further diluted to inoculate wells of a 96-well plate, containing an antibiotic dilution gradient, with  $5 \times 10^5$  cells each. The plate was grown

statically at 37 °C for 24 h. To determine the MIC the plates were visually inspected for growth and by reading the optical density (OD<sub>600</sub>) with a BioTek plate reader. To determine the MBC,  $10^{-1}$ – $10^{-3}$  dilutions of cell suspensions exposed to the antibiotic dilution gradients were plated on LB plates and incubated at 37 °C for 24 h. The number of colony forming units (CFU) per dilution was recorded. As a positive control, cells suspensions treated similarly but without exposure to the compounds were used. The MBC was calculated using the formula:

$$\text{MBC} = \frac{\text{No. of CFU in a pspecific concentration}}{\text{No. of CFU in the postive control}} \times 100$$

where number of CFU in a specific concentration is the average CFU of each dilution at each concentration tested. A concentration was considered bactericidal if it killed 99.9% of the bacterial population [14].

### Minimum biofilm inhibitory concentration (MBIC) assay

Assays were performed as previously described [18]. Briefly, an overnight culture was diluted 100-fold and incubated at 37 °C for 3 h to reach mid-log phase. The subculture of *S. aureus* was prepared by diluting the overnight culture in 1:1000 ( $\sim 10^5$  CFU ml<sup>-1</sup>) into TSB. Wells of a 96-well plate were used to serially diluted the desired compound in TSB and then inoculated with  $5 \times 10^6$  cells each and grown statically at 37 °C for 24 h. The wells were then rinsed twice with PBS (pH 7.2, 0.8% NaCl, 0.02% KCl, 0.17% Na<sub>2</sub>HPO<sub>4</sub>, 0.8% KH<sub>2</sub>PO<sub>4</sub>) and then followed with the resazurin cell variability assay below.

### MBEC assay

Assays were performed as previously described [18]. Briefly, an overnight culture was diluted 100-fold and incubated at 37 °C for 3 h to reach mid-log phase. The subculture of *S. aureus* was prepared by diluting the overnight culture in 1:1000 ( $\sim 10^5$  CFU ml<sup>-1</sup>) into TSB. Wells of a 96-well plate were inoculated with  $5 \times 10^6$  cells each and grown statically at 37 °C for 24 h. The media was then removed from each well. Then serial dilutions of the desired compound in TSB were added to the wells and grown statically at 37 °C for 24 h. The wells were then rinsed twice with PBS (pH 7.2, 0.8% NaCl, 0.02% KCl, 0.17% Na<sub>2</sub>HPO<sub>4</sub>, 0.8% KH<sub>2</sub>PO<sub>4</sub>) and then followed with the resazurin cell variability assay.

### Resazurin cell viability assay

Viable cells with active metabolism reduce resazurin into fluorescent resorufin [19]. To the washed wells, 20 µl of

CellTite-Blue (Promega) was added with 100  $\mu$ l of PBS and incubated at 37 °C for 1 h. Fluorescence was measured (530/25 nm excitation and 590/35 nm emission) using a BioTek Synergy plate reader. Wells that had the same fluorescence as a resazurin-only control were considered to have no cell viability.

### Checkerboard assay

Checkerboard assays in 96-well format were performed as previously described [20]. Briefly, the inoculum was prepared as for the MIC testing above and added to a two-dimensional gradient of the compound of interest. Plates were grown statically at 37 °C for 24 h then visually inspected for growth. The fractional inhibition concentration (FIC) index is calculated using the formula

$$\text{FIC Index} = \frac{C_1}{\text{MIC}_1} + \frac{C_2}{\text{MIC}_2}$$

where MIC<sub>1</sub> and MIC<sub>2</sub> are the MICs of antibiotics 1 and 2 alone, respectively, and C<sub>1</sub> and C<sub>2</sub> are the concentrations of antibiotics 1 and 2, respectively, when at the combined MIC. When the FIC index was  $\leq 0.5$  the antibiotic combination was considered to be synergistic; when the FIC index was  $> 0.5$  and  $\leq 4.0$  the effect of the two antibiotics was considered to be additive. Finally, when the FIC index was  $\geq 4.0$ , the antibiotic combination was defined as antagonistic [21].

### Hemolysis assay

Hemolytic activity on ovine erythrocytes was assessed as previously described [22, 23]. Briefly, ovine erythrocytes were gently pelleted by centrifugation and washed thrice with PBS then resuspended in PBS at a 1:5 dilution. In 96-well format, 100  $\mu$ l erythrocyte suspension was added to a dilution gradient of the desired compound and incubated statically at 37 °C for 1 h. Triton X-100 at 0.1% was included as a positive control and a dimethyl sulfoxide (DMSO) concentration gradient as a negative control. Erythrocytes were pelleted and the absorbance of the supernatant was measured at 540 nm. The percentage of lysis was calculated as follows:

$$\% \text{Lysis} = \frac{X - B}{T - B} \times 100\%$$

where B is the A<sub>540 nm</sub> of the negative DMSO control, T is the A<sub>540 nm</sub> of the positive Triton X-100 control, and X is the A<sub>540 nm</sub> of the analyzed sample. A<sub>540 nm</sub>  $> 40\%$  hemolysis was considered as high percent hemolysis and 5–10% hemolysis was considered as low percent hemolysis for a given compound [24].

## Synthesis and characterization of compounds 1–12

Reagents and solvents were used as purchased without further purification. Thin layer chromatography (TLC) was conducted with 0.25 mm F<sub>254</sub> silica gel on aluminum plates and visualization was conducted with 254 nm UV light. The purification of all compounds was carried out over flash grade-silica gel and fractions combined according to TLC. Solvent was removed under reduced pressure. The <sup>1</sup>H (300 MHz) and <sup>13</sup>C NMR (125 MHz) spectra were collected on a Bruker Avance-300 console and were referenced to solvent (CDCl<sub>3</sub> = 7.24 ppm). The complete synthetic procedures and characterization data for compounds 1–12 are included in the electronic supplementary material.

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**Author contributions** NM designed and performed synthesis of the compounds, HHT tested the activity of the compounds. NM and HHT interpreted the data and wrote the manuscript. AMH supervised and edited the final version of the manuscript. STC and JLS conceived the research approach, contributed to writing and edited the final version of the manuscript.

### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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## References

- Munita JM, Shelburne S, Greenberg DE, Arias CA. The growing threat of antimicrobial resistance. *Tex Med*. 2017;113:48–52.
- Public Health Agency of Canada. Canadian antimicrobial resistance surveillance system 2017 Report. 2017.
- Chambers HF, DeLeo FR. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. *Nat Rev Microbiol*. 2009;7:629–41.
- Stryjewski ME, Corey GR. Methicillin-resistant *Staphylococcus aureus*: an evolving pathogen. *Clin Infect Dis*. 2014;58:S10–S19.
- Miller LG, Kaplan SL. *Staphylococcus aureus*: a community pathogen. *Infect Dis Clin North Am*. 2009;23:35–52.
- Akil N, Muhlebach MS. Biology and management of methicillin resistant *Staphylococcus aureus* in cystic fibrosis. *Pediatr Pulmonol*. 2018. <https://doi.org/10.1002/ppul.24139>
- Gangell C, et al. Inflammatory responses to individual microorganisms in the lungs of children with cystic fibrosis. *Clin Infect Dis*. 2011;53:425–32.
- Pena Amaya P, et al. Molecular epidemiology of methicillin-resistant *Staphylococcus aureus* in cystic fibrosis patients from Argentina. *Microb Drug Resist*. 2017. <https://doi.org/10.1089/mdr.2017.0162>



9. Muhlebach MS. Methicillin-resistant *Staphylococcus aureus* in cystic fibrosis: how should it be managed? *Curr Opin Pulm Med*. 2017;23:544–50.
10. Harvey AL, Edrada-Ebel R, Quinn RJ. The re-emergence of natural products for drug discovery in the genomics era. *Nat Rev Drug Discov*. 2015;14:111–29.
11. Heihachiro T, Ushio S, Shoji S. Biosynthesis of natural products. VI) Biosynthesis of usnic acid in lichen's (1). A general scheme of biosynthesis of usnic acid. *Chem Pharma*. 1969;17:2054–60.
12. Sarkar R, Mittal N, Sorensen J, Sen T. A comparison of the bioactivity of usnic acid versus methylphloracetophenone. *Nat Prod Commun*. 2018;13:1673–6.
13. Pena Amaya P, et al. Molecular epidemiology of methicillin-resistant *Staphylococcus aureus* in cystic fibrosis patients from Argentina. *Microb Drug Resist*. 2018;24:613–20.
14. Pankey GA, Sabath LD. Clinical relevance of bacteriostatic versus bactericidal mechanisms of action in the treatment of Gram-positive bacterial infections. *Clin Infect Dis*. 2004;38:864–70.
15. Brochado AR, et al. Species-specific activity of antibacterial drug combinations. *Nature*. 2018;559:259–63.
16. Sahuquillo Arce JM. In vitro activity of linezolid in combination with doxycycline, fosfomycin, levofloxacin, rifampicin and vancomycin against methicillin-susceptible *Staphylococcus aureus*. *Rev Esp Quimioterap*. 2006;19:252–7.
17. Clinical and Laboratory Standards Institute CLSI. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard. 9th ed. CLSI; Wayne, USA, 2012.
18. Zhang E, et al. Synthesis and bioactivities study of new antibacterial peptide mimics: the dialkyl cationic amphiphiles. *Eur J Med Chem*. 2018;143:1489–509.
19. Evie IM, Dickson AJ, Elvin M. Metabolite profiling of mammalian cell culture processes to evaluate cellular viability. In: Gilbert DF, Friedrich O, editors. *Cell viability assays: methods and protocols*. New York: Springer; 2017. p. 137–52.
20. Garcia Lynne S. *Clinical microbiology procedures handbook*, vols. 1–3. 3rd ed. ASM Press, Washington, DC, 2016.
21. van Belkum A, et al. Meropenem/colistin synergy testing for multidrug-resistant *Acinetobacter baumannii* strains by a two-dimensional gradient technique applicable in routine microbiology. *J Antimicrob Chemother*. 2015;70:167–72.
22. Hogan AM, et al. Competitive fitness of essential gene knock-downs reveals a broad-spectrum antibacterial inhibitor of the cell division protein FtsZ. *Antimicrob Agents Chemother*. 2018;62:e01231–18.
23. Selin C, et al. A pipeline for screening small molecules with growth inhibitory activity against *Burkholderia cenocepacia*. *PLoS One*. 2015;10:e0128587.
24. Sperandio D, et al. Cell-associated hemolysis activity in the clinical strain of *Pseudomonas fluorescens* MFN1032. *BMC Microbiol*. 2010;10:124.