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Letter

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Meridianin D analogues display antibiofilm activity against MRSA, increase colistin efficacy in Gram-negative bacteria

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ABSTRACT: In the last 30 years, development of new classes of antibiotics has slowed, increasing the necessity for new options to treat multi-drug resistant bacterial infections. Development of antibiotic adjuvants that increase the effectiveness of currently available antibiotics is a promising alternative approach to classical antibiotic development. Reports of the ability of the natural product meridianin D to modulate bacterial behavior have been rare. Herein, we describe the ability of meridianin D to inhibit biofilm formation of methicillin-resistant *Staphylococcus aureus* (MRSA) and increase the potency of colistin against colistin-resistant and sensitive Gram-negative bacteria. Analogues were identified that are capable of inhibiting and dispersing MRSA biofilms and lowering the colistin MIC to below the CLSI breakpoint against *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Escherichia coli*.

Antibiotic resistance is quickly becoming one of the largest threats to human health. Without the development of new strategies to defeat antibiotic resistance it is predicted that 10 million people will die from multi-drug resistant (MDR) bacterial infections by 2050.[1] Compounding the problem, resistant isolates to the two classes of antibiotics introduced most recently in the clinic, cyclic lipopeptides and oxazolidinones, were observed within five years of clinical use.[2] An alternative strategy to new antibiotics is to develop adjuvants that intercept the pathways responsible for resistance to clinically relevant antibiotics. Bacteria are capable of avoiding antibiotic treatment in many ways, including biofilm formation. Biofilms are highly organized surface-associated communities that are encased in an extra-cellular polymeric substance (EPS). Bacteria within a biofilm are upwards of 1000-fold more resistant to antibiotic treatment and reach a higher cell density than their planktonic counterparts, increasing the chances of horizontal gene transfer.[3,4] In many cases, bacteria also evade antibiotic treatment by acquiring resistance elements in small gene vectors. One example is the spread of the plasmid-borne *mobile colistin resistance-1 (mcr-1)*[5,6] gene, which likely evolved from overuse of colistin as a food additive in animal husbandry. Selective pressure has also been applied by the resurgence of colistin treatment clinically as an antibiotic of last resort in MDR Gram-negative bacterial infections.[7] The emergence of these colistin resistant strains brings us closer to a post-antibiotic world., exemplified when a MDR strain of *Klebsiella pneumoniae* was observed clinically in 2016 that was resistant to all clinically available antibiotics.[8]

Natural products from marine sponges have long been a rich source of molecules that display a myriad of biological activities. The meridianins are one such example of a family of structurally related marine natural products. These secondary metabolites were first reported in 1998 after being isolated from the marine invertebrate *Aplidium meridianum* near the South Georgia Islands.[9] The family of meridianins and their respective derivatives have shown diverse biological activities including kinase inhibition[10,11], adipogenesis inhibition[12], antitumor activity[13], and anti-malarial activity.[14] Reports of antibacterial activity of these compounds, however, have been scarce and fragmentary. These reports have been limited to antimicrobial activity against *Staphylococcus aureus*[14], *Mycobacterium tuberculosis*[15] and an unidentified sympatric marine Antarctic bacterium.[16] Despite these limited reports, we posited that the meridianins and their analogues would possess the ability to control bacterial behavior based on the shared structural features of the meridianins with the desformylflustrabromine (dFBr)[17], 2-aminopyrimidine (2-AP)[18], and oroidin[19,20] analogues that were previously shown by our group to possess antibiofilm activity against methicillin-resistant *S. aureus* (MRSA) (**2** and **3**) and the ability to lower the colistin minimum inhibitory concentration (MIC) against Gram-negative bacterial pathogens carrying the *mcr-1 plasmid* (**4**) (Figure **1**). Herein, we report the antibiofilm and antibiotic activity of analogues based on the meridianin scaffold against *S. aureus*. Moreover, we report the ability of meridianin analogues to lower the MIC of colistin against both colistin-sensitive and resistant strains of Gram-negative bacteria.

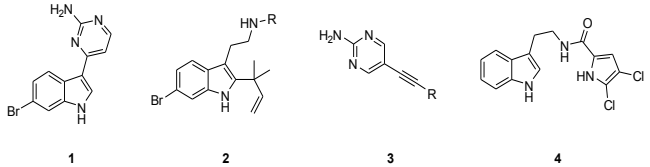
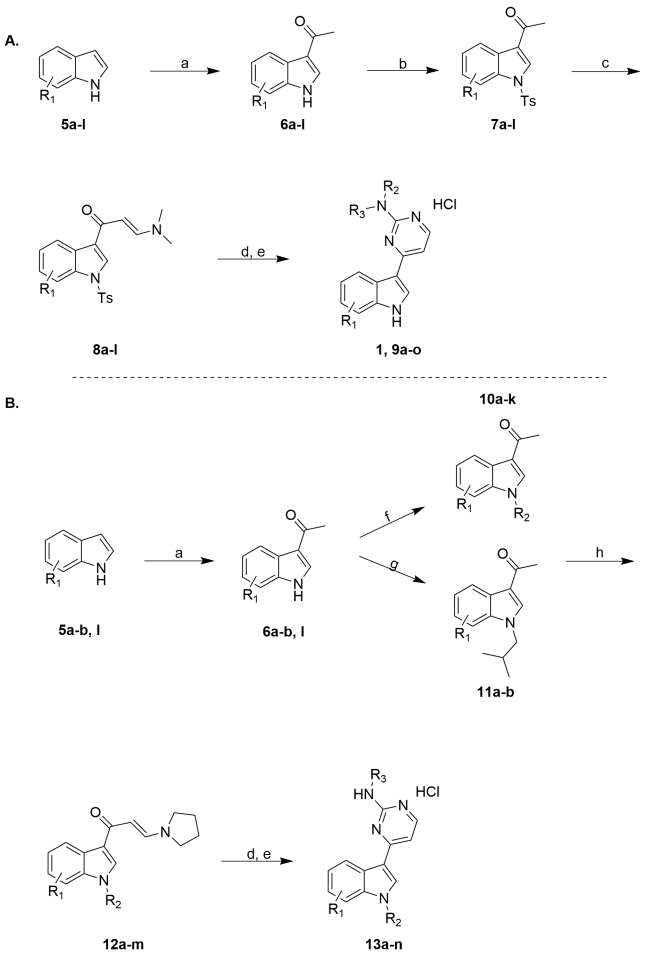


Figure 1. Structures of meridianin D (compound **1**), desformylflustramine analogues (compound **2**), 2-AP analogues

(compound **3**), and oroidin analogue (compound **4**) previously shown to control bacterial behavior.



Scheme 1. Synthetic route to compounds **1, 9a-o (A)** and **13a-m (B)**: (a) Acetyl chloride, $SnCl_4$, toluene, 0 °C to rt, 2 h; (b) p-toluenesulfonyl chloride, triethylamine, 4-dimethylaminopyridine, DCM, rt, 16 h; (c) DMF-DMA, 110 °C, 3 h; (d) Guanidine derivative, K_2CO_3, 2-methoxyethanol, reflux, 16 h (e) MeOH/HCl (f) Dimethyl sulfate or R_2X, 50% NaOH, DCM, 16 h; (g) 1-bromo-2-methylpropane, K_2CO_3, acetone, reflux, 16 h; (h) pyrrolidine, DMA, 80 °C, 1 h (i) DMF, 110 °C, 4 h.

To begin the structure-activity relationship (SAR) study of the meridianin molecules, we synthesized meridianin D **1** to establish its biological activity. Attempts to synthesize meridianin D following the procedure described by Jiang *et al.*[21] were unsuccessful as problems described by Simon *et al.*[22] were encountered. Therefore, we applied the synthetic approach outlined by Bredereck to access meridianin D and analogues (Scheme **1A**).[11, 22-24] To begin, commercially available substituted indole derivatives were acylated at the 3-position using acetyl chloride and tin chloride in toluene to yield compounds **6a-l**. The indolic nitrogen of compounds **6a-l** was subsequently protected using *p*-toluenesulfonyl chloride (TsCl), triethylamine, and 4-dimethylaminopyrimidine (DMAP) in DCM. Next, the enaminone derivatives were prepared by reacting compounds **7a-l** with DMF/dimethylformamide-dimethylacetal (DMF-DMA) at 110° C for 4 hours. Cyclization and deprotection of the enaminone in 2-methoxyethanol using potassium carbonate and guanidine hydrochloride or commercially available substituted guanidine derivatives yielded compounds **1, 9a-o**.

Compounds were first assessed for their ability to inhibit MRSA biofilm formation. Meridianin D (**1**) returned an IC_{50} value of 87.4±4.0 μM (Table **1**), where the IC_{50} value is defined as the concentration at which a compound inhibits 50% of biofilm formation. This result confirmed our hypothesis that the meridianin natural products would be capable of inhibiting MRSA biofilm formation. The 4-, 5-, and 7-bromo analogues were assayed to investigate the effect that the substitution of the bromine atom had on the compound's antibiofilm activity. The 4-bromo (compound **9k**) and 7-bromo (compound **9j**) analogues displayed reduced antibiofilm activity, with IC_{50} values of >100 and 99.8±15.2 μM respectively. The 5-bromo analogue, compound **9a**, displayed increased biofilm inhibitory activity with an IC_{50} value of 17.9±2.2 μM.

After identifying the 5-bromo and 6-bromo analogues as the most active derivatives, various substitutions on the indole ring were prepared while preserving the 2-AP ring to probe the promiscuity of the indole substitution. Of these analogues, the debromo analogue **9o**, 5-methyl **9n**, 5-fluoro **9l** and 6-fluoro **9m** analogues all displayed no antibiofilm activity with IC_{50} values of >100 μM. The 6-chloro analogue **8i** displayed comparable activity to meridianin D while the 5-chloro analogue **9h** showed reduced activity compared to the 5-bromo analogue (**8b**). Finally, the 6-iodo analogue **9f** displayed increased activity compared to meridianin D, with an IC_{50} of 42.5±8.1 μM and the 5-iodo analogue **9g** displayed reduced activity compared to the 5-bromo analogue (**9a**), with an IC_{50} of 49.3±5.1 μM.

Upon identifying compound **9a** as the most active compound from this series, the effect of alkylation of the exocyclic amine of the 2-AP ring on the antibiofilm activity was investigated. Cyclization with a substituted guanidine in place of guanidine hydrochloride in the final step of the synthesis yielded methyl (compound **9e**), ethyl (compound **9d**), dimethyl (compound **9c**) and benzyl (compound **9b**) analogues. Methyl (**9e**) and ethyl (**9d**) substitutions on the 2-AP ring decreased activity, delivering compounds with IC_{50}'s of 28.9±2.3 and 24.6±0.7 μM respectively. These substitutions also made these analogues more toxic to planktonic bacterial growth with MICs of 100 μM for the methyl and 50 μM for the ethyl, compared to 200 μM for the unsubstituted parent. Dimethyl substitution (**9c**) of the 2-AP was still less active than the unsubstituted 2-AP but showed similar activity compared to the mono methyl substituted analogue, with an IC_{50} of 23.4±1.8 μM. Placement of a benzyl group at the exocyclic amine of the 2-AP ring, compound **9b**, significantly increased the activity of the compound, lowering the IC_{50} to 9.62±1.4 μM. The reduction in IC_{50} for the benzyl analogue was coupled with a significant increase in toxicity, reducing the MIC from 200 μM for compound **9a** to 6.25μM for the benzyl substituted analogue **9b**. Observing a significant decrease in MIC, the antibiotic activity of compound **9b** was explored against a small panel of Gram-positive pathogens. The benzylated analogue returned an MIC value of 6.25 μM (2.60 μg/mL) against two additional *S. aureus* isolates and an MIC of 25 μM (10.4 μg/mL) against a strain of vancomycin resistant *Enterococcus faecium* (VRE). No significant difference in compound MIC value was observed when compounds **9b**, **9d**, **9n** and **9o** were tested in the presence or absence of 0.01% triton X-100 against MRSA 43300.

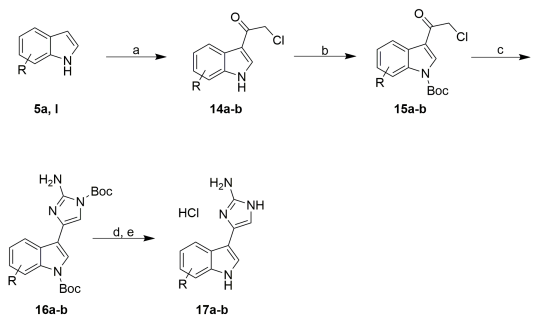
Table 1. IC_{50} and MIC values for compounds **1, 9a-g, 13a-f**. All values displayed against MRSA 43300. Full biofilm inhibition results can be found in the supporting information.

Compound	R_1	R_2	R_3	MIC (μM)	IC_{50} (μM)
1	6-Br	H	H	>200	87.4±4.0
9a	5-Br	H	H	200	17.9±2.2
9b	5-Br	Bn	H	6.25	9.62±1.4
9c	5-Br	Me	Me	100	23.4±1.8
9d	5-Br	Et	H	50	24.6±0.7
9e	5-Br	Me	H	100	28.9±2.3
9f	6-I	H	H	>200	42.5±8.1
9g	5-I	H	H	200	49.3±5.1
13a	6-Br	n-Pr	H	200	34.3±6.8
13b	5-Br	n-Bu	H	50	38.1±2.8
13c	6-Br	Me	H	200	59.6±2.3
13d	6-Br	Et	H	200	64.0±8.8
13e	5-Br	Et	H	200	66.2±7.6
13f	5-Br	Pr	H	100	69.3±3.6

With compounds **1**, **9.5**, and **9f** established as leads displaying minimal inherent toxicity, the effect of alkylation of the indole nitrogen on antibiofilm activity was next interrogated. Synthesis of compounds **13a-m** was adapted from a previous disclosure by Simon *et al.* (Scheme **1B**).[22] Acylation of a substituted indole at the 3 position proceeded as previously reported. Compounds **l** and **6a-b** were then alkylated using dimethyl sulfate or the desired alkyl halide with tetrabutylammonium bromide as a phase transfer catalyst in a biphasic mixture of dichloromethane and 50% NaOH. Alkylation with isobutylbromide failed under these conditions and required compounds to be refluxed in acetone with isobutyl bromide and potassium carbonate to yield the desired product. Compounds **10a-k, 11a-b** were then transformed into enaminones **12a-m** by stirring a mixture of DMA with pyrrolidine for 1 hour at 80° C followed by the addition of a solution of the appropriate n-alkylated acetyl indole (compounds **10a-k**, **11a-b**) dissolved in DMF and stirring the reaction at 110° C for 3 hours. Finally, cyclization of the enaminone with guanidine hydrochloride or commercially available substituted guanidine derivatives proceeded in 2-methoxyethanol at reflux with potassium carbonate for 16 hours to yield compounds **13a-m**. Methylation of the indolic nitrogen of the 6-bromo derivative, compound **13c**, improved the IC_{50} value to 59.6±2.3 μM (Table **1**) from 87.4±4.0 μM (compound **1**). Ethylation of the indole nitrogen, compound **13d**, displayed no improvement compared to compound **13c**, but the propyl derivative, compound **13a**, displayed increased activity with an IC_{50} of 34.3±6.8 μM. The butyl derivative, compound **13j**, displayed no antibiofilm activity with an IC_{50} of >100 μM. Interestingly, alkylation of the indole nitrogen of the 5-bromo analogues followed a different activity trend than the 6-bromo analogues. Substitution with a methyl group, compound **13g**, and n-pentyl group, compound **13h**, abolished antibiofilm activity (IC_{50}'s >100 μM). Ethyl and n-propyl derivatives, compounds **13e** and **13f** respectively, showed decreased activity compared to the 5-bromo derivative with a free indolic nitrogen with IC_{50} values of 66.2±7.6 μM and 69.3±3.6 μM respectively. The n-butyl derivative, compound **13b**, displayed an IC_{50} of 38.1±2.8 μM, but was more toxic than other analogues with an MIC of 50 μM indicating that it may be acting via a toxic mechanism to prevent biofilm formation. Branching of the alkyl chains with isobutyl substitutions on both the 5-bromo and 6-bromo analogues, compounds **13k** and **13l** respectively, abrogated antibiofilm activity with both N-isobutyl derivatives both displaying IC_{50} values of >100 μM. Next, the 6-iodo analogue, compound **9f**, was alkylated off the indolic nitrogen with a propyl group to yield compound **13m**. Again, it was observed that the indolic alkyl substituent and halogen substitution of the indole did not correlate to each other as compound **13m** displayed no antibiofilm activity. Finally, concurrent alkylation of the indolic nitrogen and the exocyclic 2-AP nitrogen, compound **13n**, abolished all antibiofilm activity.

With a panel of 2-AP analogues in hand, we turned our interests towards replacement of the 2-AP ring with a 2-aminoimidazole (2-AI). Previously, 2-AIs have shown excellent antibiofilm activity against a wide variety of pathogenic bacteria, including MRSA.[25-27] The synthesis of the 2-AI analogues proceeded with the acylation of a substituted indoles **5a** and **5l** with chloroacetyl chloride in toluene (Scheme **2**). The indolic nitrogen of compounds **14a-b** then were Boc protected to yield compounds **15a-b**. Cyclization of the α-chloroketone with Boc guanidine and sodium iodide in DMF yielded compounds **16a-b**. Subsequent Boc deprotection using TFA in DCM at 0° C for 16 hours delivered the 2-AI derivatives **17a-b**. Interestingly, both compounds **17a-b** were toxic to planktonic bacterial growth at 60 μM under the conditions of the biofilm inhibition assay and displayed no antibiofilm activity below this concentration. Both compounds did display moderate antimicrobial activity returning MICs of 25 μM (7.8 μg/mL) against our test MRSA strain.

Scheme 2. Synthetic route to compounds **17a-b**: (a) (i) Chloroacetylchloride, toluene, 60 °C, 2 h (ii) MeOH, H_2O, rt, 1 h (b) Boc-anhydride, 4-dimethylaminopyridine, THF, rt, 4 h (c) Boc-guanidine, sodium iodide, DMF, rt, 48 h (d) 30% Trifluoroacetic acid, DCM, 0 °C to rt, 16 h (e) MeOH/HCl.



Noting that various meridianin derivatives were capable of inhibiting MRSA biofilm formation, we next investigated whether they were capable of dispersing pre-formed MRSA biofilms. Interestingly, the alkylated 2-AP analogues displayed the greatest activity against pre-formed MRSA biofilms, with the methyl (compound **9e**) and ethyl (compound **9d**) derivatives displaying EC_{50} values, the concentration at which a compound disperses 50% of a preformed biofilm, of 73.1±2.4 and 75.8±5.8 μM respectively (Table **2**). It does not appear that the ability to disperse pre-formed biofilms is related to increased toxicity, because the more toxic compound **9b** displayed only 26.2% dispersion at 80 μM. Interestingly, dimethylation of the exocyclic amine (compound **9c**) and alkyl substituents on the indolic nitrogen (compounds **13a-b**) did not impart the ability to disperse pre-formed biofilms with all displaying EC_{50} values of greater than 160 μM. Compound **9a** returned an EC_{50} value of 138.4±15.2 μM, demonstrating its ability to inhibit and disperse MRSA biofilms.

After confirming the ability of multiple analogues of meridianin D to disperse pre-formed MRSA biofilms, the most active compounds (**9a**, **d**, and **e**) were tested for synergy with vancomycin at a concentration in which vancomycin does not affect pre-formed biofilms. This concentration was determined to be 19.0 μg/mL (supplemental information) against MRSA 43300, which is 19 times its MIC. Accordingly, each compound was tested with 19 μg/mL of vancomycin to investigate whether the compounds would show a synergistic effect with vancomycin and disperse pre-formed biofilms at a lower concentration. Compound **9a** displayed a 33% reduction in EC_{50} value (Table **3**) when combined with a non-active concentration of vancomycin against pre-formed biofilms. Other compounds tested that were capable of dispersing pre-formed MRSA biofilms did not display a significant reduction in EC_{50} value when combined with vancomycin.

Table 2. EC_{50} values with and without vancomycin for active anti-biofilm analogues. All values displayed against MRSA 43300

Compound	EC_{50} (μM)	EC_{50} (μM)+ vancomycin
9a	138.4±15.2	92.1±17.5
9e	73.1±2.4	71.4±6.0
9d	75.8±5.8	66.5±7.5
17a	101.5±0.5	>100
17b	101.0±3.9	>100
9b	>80	>80
9c	>160	N/A
13a	>160	N/A
13b	>160	N/A

In a recent disclosure,[19] synergy was found in concomitant treatment with colistin (polymyxin E) and compound **4** (Figure **1**). This combination successfully disarmed colistin resistance in multiple strains carrying the *mcr-1* plasmid-borne resistance gene. To our knowledge, there is no precedent for indole-containing compounds directly modulating polymyxin defense pathways in Gram-negative pathogens. Holistically, both compound **4** and meridianin D (compound **1**) are small, indole-derived compounds with an additional nitrogenous heterocyclic appendage. Given their semblance, we postulated that compound **1** and its analogues potentially had the ability to modulate colistin resistance in Gram-negative bacteria.

Table 3. Colistin potentiation by select compounds against Gram-negative bacteria, colistin MIC(fold reductions). Concentrations are shown in μg/mL.

	Colistin MIC (μg/mL)	Colistin+ 1	Colistin+ 9d	Colistin+ 13f
***E. coli* ATCC 25922^{mcr-1}**	8	4 (2)	0.5 (16)	2 (4)
***E. coli* ATCC 25922^{parent}**	0.5	0.5 (0)	0.0625 (8)	0.03125 (16)
***A. baumannii* 17978^{mcr-1}**	16	0.5 (32)	0.125 (128)	4 (4)
***A. baumannii* 17978^{parent}**	1	0.5 (2)	0.0625 (16)	0.0625 (16)
***A. baumannii* 4106**	1024	16 (64)	2 (512)	16 (64)
***K. pneumoniae* B9**	512	16 (64)	1 (512)	1 (512)
***A. baumannii* 5075**	1	0.25 (4)	0.0625 (16)	0.0078 (128)
***K. pneumoniae* ATCC 2146^{NDM-1}**	1	0.5 (2)	0.0625 (16)	0.0625 (16)

To this end, *Acinetobacter baumannii* ATCC 17978^{mcr-1} was chosen as a test strain.[28] As summarized in table **3**, we indeed found activity with multiple meridianin D analogues demonstrating synergy with colistin in a diverse panel of bacterial isolates comprised of both colistin-resistant and colistin-sensitive *A. baumannii, K. pneumoniae,* and *Escherichia coli*. Of note, across all strains, all analogues with the exception of compounds **13a, 17a,** and **17b** had no inherent toxicity, with MICs of >200 μM. Accordingly, all analogues were dosed at 60 μM unless otherwise noted (Supporting Information).

Cross referencing activity in these eleven strains, limited activity is seen in compounds with varied indole halogenation while alkylation of the indolic nitrogen increased activity in colistin-sensitive isolates. Alkylation of the exocyclic amine of the 2-AP delivered compounds with enhanced synergistic activity against colistin resistant strains while retaining synergy in colistin-sensitive

strains, with compound **9d** being a nearly universal modulator. Compound **9d** outperforms all other analogues in both *A. baumannii* 17978^{mcr-1} and *E. coli* 25922^{mcr-1} with 128 and 16-fold reductions in colistin MIC, respectively.

A. baumannii 3941/4106 and *K. pneumoniae* C3/B9 are primary clinical isolates that contain genomically encoded colistin resistance. Such strains typically possess much higher colistin MICs than their *mcr-1* counterparts. All four strains return colistin MICs of 512 μg/mL or greater, well exceeding the 4 μg/mL Clinical & Laboratory Standards Institute (CLSI) breakpoint. Compounds **9b** and **9d** recover a breakpoint MIC in *A. baumannii* 3941, while compound **9d** delivers a 512-fold reduction in *A. baumannii* 4106, reducing the MIC to 1 μg/mL. Colistin sensitivity is also re-established in *K. pneumoniae* C3 by compounds **9b**, **9d**, and **9e**, all producing 512-fold reductions, and this same activity is seen in *K. pneumoniae* B9 with compounds **9d**, and **9e**, and **13f**. No significant difference in colistin MIC value was observed when compounds **9b**, **9d**, **9n** and **9o** were tested in the presence or absence of 0.01% triton X-100 against *A. baumannii* 4106 and *K. pneumoniae* B9.

A select cohort of analogues were capable of increasing colistin sensitivity in strains with no inherent resistance to the antibiotic. In the parent *A. baumannii* 17978 strain, **9d** is equipotent with **9b, 13f**, **13b**, and **13k**, all generating a 16-fold reduction in colistin MIC. A 16-fold reduction in parent strain *E. coli* 25922 is observed by **13b**, **13f**, and **13k**, while **9d** shows an 8-fold reduction. A total of six compounds (compounds **9b**, **9d**, **9e, 13a**, **13b**, and **13k**) produced a colistin MIC of 0.0625 μg/mL (16-fold reduction) in *A. baumannii* ATCC 19606, while a more impressive 128-fold reduction (0.0078 μg/mL) was achieved with compound **13f** in *A. baumannii* ATCC 5075. Compound **13f** was equipotent with compound **9d** in *K. pneumoniae* ATCC 2146^{NDM-1}, returning a final MIC of 0.0625 μg/mL (16-fold reduction).

In conclusion, after identifying the potential of the natural product meridianin D (compound **1**) to control MRSA biofilm formation, a panel of analogues were synthesized in an effort to augment activity. Structural modification of the meridianin D core delivered molecules that modified both Gram-positive and Gram-negative bacterial defense mechanisms. In many cases, modification of key positions of the scaffold amended bacterial behavior in divergent manners. Of note, compound **9a** inhibited and dispersed MRSA biofilms as a stand-alone treatment. Furthermore, the EC_{50} of compound **9a** was reduced by 33.4% in concomitant treatment with vancomycin dosed at levels that do not perturb pre-formed MRSA biofilms. Alkylation of the exocyclic amine of compound **9a** with ethyl (**9d**) or methyl (**9e**) groups delivered analogues with increased ability to disperse MRSA biofilms; however, they do so with a tandem increase in toxicity and diminished synergy with vancomycin. Benzylated analogue **9b** was the most potent MRSA biofilm inhibitor with an IC_{50} of 9.62±1.4 μM, but did not disperse pre-formed MRSA biofilms. Interestingly, compound **9b** also returned modest antimicrobial activity against a small panel of Gram-positive bacteria. Furthermore, we established that certain meridianin analogues increase the efficacy of colistin against Gram-negative bacteria. Compound **9d,** bearing an ethyl substitution on the exocyclic nitrogen of the 2-AP ring, displayed the greatest range of colistin synergy across a panel of colistin-sensitive and colistin-resistant strains of Gram-negative bacteria which included highly resistant primary clinical isolates of chromosomally encoded resistance, strains of *E. coli* and *A. baumannii* harboring the *mcr-1* gene, and a strain of *K. pneumoniae* carrying the New Dehli metallo-beta-lactamase-1 (*NDM-1*) gene. Compounds **13a**, **13b**, **13f**, and **13k** which bear an alkylation on the indolic nitrogen displayed equivalent or greater potentiation of colistin activity against some strains, but did not display the broad-spectrum potentiation of colistin activity of compound **9d**. Mechanistic studies and additional structural modifications in an effort to increase activity of the analogues are currently underway.

ASSOCIATED CONTENT

Supporting Information

Compound characterization for all novel compounds, ^{1}H and ^{13}C NMR spectra, biofilm, colistin repotentiation and full in-vitro testing data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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