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Letter

# Novel Potentiators for Vancomycin in the Treatment of Biofilm-Related MRSA Infections via a Mix and Match Approach

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Supporting Information

**ABSTRACT:** A library of 52 hamamelitannin analogues was synthesized and investigated for its ability to potentiate the effect of vancomycin toward *Staphylococcus aureus* biofilms. Several compounds were found to effectively increase the susceptibility of staphylococcal biofilms toward this glycopeptide. The most active analogue identified in this study showed an EC<sub>50</sub> value of 0.26  $\mu$ M.



KEYWORDS: Antibiotic potentiators, Staphylococcus aureus, hamamelitannin analogues, quorum sensing, biofilms

Staphylococcus aureus (S. aureus) is an opportunistic pathogen and a leading cause of bacterial infection.<sup>1</sup> Methicillin-resistant Staphylococcus aureus (MRSA) represents a nosocomial as well as a communal menace, and antimicrobial resistance (AMR) is eroding the clinical utility of existing antibiotics. Additionally, biofilm formation represents a significant impediment to treatment since it may worsen antimicrobial resistance to traditional antibiotics and impede host immune responses.<sup>2</sup> Despite a recent surge, the discovery of novel antibacterial classes proves challenging.<sup>3</sup> Hence, there is a significant and urgent need for additional therapies to combat bacterial pathogens.

Although the ability to mass-produce antimicrobial drugs constitutes a monumental scientific achievement of the twentieth century, conventional antibiotics inherently impose selective pressure on bacteria and cause an acceleration of resistance development. Bacteria have demonstrated resistance mechanisms to virtually every antibiotic introduced by the medical community. In that light, it may be argued that next to the traditional strategy of antibiotic discovery, the development of alternative approaches should also be considered to combat bacterial infections in the future.

One such strategy is the use of combinations of drugs, a paradigm that is clinically proven in many areas of medicine (e.g., in cancer chemotherapy and infectious diseases such as tuberculosis and HIV/AIDS). The pairing of an antibiotic with a nonantibiotic molecule that potentiates the activity of the former is extensively discussed in the literature.<sup>4–7</sup> Although

not possessing growth-inhibitory activity by themselves, smallmolecule "potentiators" can reduce antibiotic use and/or lessen the likelihood of resistance development. The prototype example is the  $\beta$ -lactamase inhibitor clavulanic acid, which "augments" the activity of the  $\beta$ -lactam antibiotic amoxicillin by inhibiting  $\beta$ -lactamases.

Kiran et al. previously identified the natural product hamamelitannin (HAM, **1**, Figure 1) as a quorum sensing inhibitor (QSI) in *S. aureus.*<sup>8</sup> The hydrolyzable tannin was later shown to increase *S. aureus* biofilm susceptibility toward vancomycin (VAN).<sup>9</sup> Recently, we demonstrated that HAM affects peptidoglycan thickness and eDNA release through the quorum sensing receptor TraP. Notably, it operates via



Figure 1. Structure of hamamelitannin (HAM, 1) and a selection of more drug-like derivatives (2, 3, 4, and 5).

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<sup>*a*</sup>Reagents and conditions: (a) 2-chlorobenzoyl chloride, PMe<sub>3</sub>, THF, rt, 16 h (57%); (b) H<sub>2</sub>N-NH<sub>2</sub>·H<sub>2</sub>O, EtOH, Δ, 5 h (82%); (c) RCOOH, EDC-HCl, DIPEA, HOBt, DMF, rt, 16 h (68–99%); (d) (AcO)<sub>2</sub>O, DIPEA, 0 °C (71%); (e) 35% TFA in H<sub>2</sub>O (59%–quant.).

nongrowth inhibiting mechanisms.<sup>10</sup> Stability issues of the QSI were believed to be detrimental for further development, which led us to search for more stable analogous potentiators,<sup>11</sup> a selection of which is shown in Figure 1 (2, 3, 4, and 5). The metabolically stable HAM analogue 2 (i.e., compound 38 in our previous publication<sup>11</sup>) was selected as a lead for further study and was shown to possess the ability to potentiate the effect of several classes of antibiotics *in vitro*. Moreover, the bisbenzamide increases the effect of antibiotics in two *in vivo* infection models.

Initial efforts to optimize the activity and stability of HAM were mainly focused on varying the 5-position of the molecule.<sup>11,12</sup> In the present study, we want to investigate structural variation at C-2'. Additionally, we synthesized hybrid analogues in which individual optimal aromatic moieties are combined into a single molecule in various ways.

To gain initial information on the structure-activity relationship (SAR) at the 2'-position, we focused on benzamides with different substituents in different positions (Scheme 1). Next to that, derivatives with longer amide moieties (i.e., derived from phenyl acetic acid and phenyl propanoic acid) were synthesized. Finally, the library was extended with nonaromatic amides (i.e., an acetamide and a cvclohexanecarboxamide derivative), which allowed us to assess the role of an aromatic ring at the 2'-side. Note that for convenience of comparison an ortho-chlorobenzamide moiety was kept at the opposite side of the molecule. The synthesis starts from the known orthogonally protected phthalimide 6 (Scheme 1).<sup>11</sup> One-pot azide reduction and acylation yielded intermediate 7 and avoided dimer formation due to attack of the amine on the phthalimide of a second molecule. Removal of the phthalimide with ethanolic hydrazine gave primary amine 8, which was used to generate a series of amide derivatives via EDC-mediated acylation with the appropriate carboxylic acid (9a-t). Acetamine 9u was synthesized according to standard conditions. Acidic hydrolysis of the acetonide afforded the final HAM analogues 10a-u.

The minimum inhibitory concentrations (MIC) of all final compounds against *S. aureus* Mu50 were higher than 500  $\mu$ M, indicating that they had no direct effect on growth in the concentrations used in the present study (data not shown). Subsequently, the HAM derivatives were tested for their *in vitro* effect on *S. aureus* biofilm susceptibility to vancomycin (VAN). VAN is considered a drug of last resort against MRSA. The biofilm susceptibility testing was done both under pretreatment

and under combination treatment regimens. In the pretreatment test, *S. aureus* Mu50 was allowed to form a biofilm in the presence of a HAM analogue, after which the biofilm was treated with VAN (20  $\mu$ g/mL). In the combination treatment setup, the bacteria were allowed to form a mature biofilm, after which VAN and a HAM derivative were administered simultaneously. *S. aureus* Mu50 is a clinical MRSA as well as a VISA strain (vancomycin-intermediate *Staphylococcus aureus*) and hence a very tenacious pathogen.<sup>13</sup> Indeed, when used alone, VAN resulted only in a minor reduction of the number of *S. aureus* Mu50 sessile cells (30 ± 14% compared to an untreated control, Table 1). In contrast, combined treatment of

 Table 1. Microbiological Evaluation of HAM Analogues with

 Differentiation at the 2'-Position

	Reduction in CFU's compared to ctrl $(\%)^{b}$		$EC_{50}$ ( $\mu$ M)	
compd <sup>a</sup>	pretreatment	combination treatment	pretreatment	combination treatment
VAN alone	30 ± 14	30 ± 14		
HAM, 1	$35 \pm 12$	$31 \pm 11$	146	165
2	$85 \pm 5^{c}$	$80 \pm 4^{c}$	0.39	7.98
10a	48 ± 9	$58 \pm 7^{c}$	79.7	41.9
10c	$39 \pm 3$	43 ± 6	n.d.	n.d.
10d	$34 \pm 18$	43 ± 19	n.d.	n.d.
10n	$38 \pm 18$	$56 \pm 12^{c}$	48.6	62.2
10o	36 ± 14	$35 \pm 21$	n.d.	n.d.
10q	$58 \pm 13^{c}$	$50 \pm 8^{c}$	68.5	76.7
10s	$35 \pm 12$	$18 \pm 22$	n.d.	n.d.
10t	$85 \pm 2^{c}$	$55 \pm 7^{c}$	9.56	34.0
10u	$70 \pm 9^{c}$	$82 \pm 11^{c}$	15.4	3.55
15	$80 \pm 12^{c}$	$81 \pm 8^{c}$	11.4	4.99
21	$65 \pm 10^{c}$	44 ± 9	n.d.	n.d.
22	$67 \pm 10^{c}$	$58 \pm 15^{\circ}$	n.d.	n.d.
23	$94 \pm 5^{c}$	$89 \pm 4^{c}$	6.32	8.42
24	$92 \pm 3^{c}$	$73 \pm 8^{c}$	13.7	20.9
25	$87 \pm 4^{c}$	$88 \pm 2^{c}$	15.1	2.34

<sup>*a*</sup>Compounds are in addition to VAN. <sup>*b*</sup>Percentage reduction in Colony Forming Units (CFU's) per biofilm when biofilms are treated with VAN alone (20  $\mu$ g/mL) or in combination with HAM or a HAM analogue (25  $\mu$ M) compared to the untreated (negative) control. <sup>*c*</sup>\*Significantly different from treatment with VAN alone (p < 0.05). n.d.: not determined.

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VAN with 2 resulted in significantly more killing of bacterial biofilm cells, both under pretreatment and under combined treatment regimens. Initially, all of the compounds with variation at the 2'-side of the molecule (10a-u) were tested in a concentration of 25  $\mu$ M. For the most interesting derivatives, the effect on biofilm susceptibility toward VAN was also tested in lower concentrations, which allowed us to determine an EC<sub>50</sub> value. The latter is defined as the concentration of the analogue needed to double the effect of VAN, as measured by the number of surviving cells. A full overview of the activities of the compounds from the 10 series is given in Table S1 (Supporting Information).

Our efforts to optimize the activity of **2** via variation of the 2'-phenyl seemed challenging. Interestingly, none of the bisbenzamides (a selection of which is given in Table 1) were exceptionally potent, with some of them being almost completely devoid of activity (e.g., **10c** and **10d**). In accordance with previous results,<sup>11</sup> increasing the distance between the two aromatic rings is detrimental for activity (exemplified by **10r** in Table S1 and **10s** in Table 1). The shorter acetamide **10u**, however, shows very good potentiating activity in the combination treatment setup (EC<sub>50</sub> = 3.55  $\mu$ M).

More pronounced truncation of 2 was realized through the synthesis of the "single-winged" 1-deoxyribose analogue 15 (Scheme 2). The known 5-azido-2,3-O-isopropylidene-D-





"Reagents and conditions: (a) LiBH<sub>4</sub>, THF, rt (44%); (b) [i] TsCl, pyr, rt, 4 h, [ii] 60 °C, 14 h (54%); (c) [i] PMe<sub>3</sub>, THF, H<sub>2</sub>O, [ii] 2-chlorobenzoic acid, EDC·HCl, DIPEA, HOBt, DMF, rt, 16 h (52%); (d) 35% TFA in H<sub>2</sub>O (85%).

ribonolactone  $11^{14}$  was reduced to diol 12 using LiBH<sub>4</sub>. Tosylate-promoted cyclization yielded tetrahydrofuran scaffold 13. Staudinger reduction of azide 13 gave the corresponding amine, which was acylated with *o*-chlorobenzoic acid and EDC as the coupling reagent. The acetal protecting group was cleaved using strong acid conditions to give final compound 15.

To shed light onto the role of the 2'-amido linker, we explored other nitrogen-based moieties to link the aromatic group to the central scaffold. Intermediate 8 served to construct analogues with alternative moieties (Scheme 3). Here again, an *ortho*-chlorobenzamide was installed in the 5-position. Treatment of amine 8 with benzenesulfonyl chloride gave sulfonamide 16, while treatment with phenylisocyanate yielded urea derivative 17. Imine formation of amine 8 with benzaldehyde and careful treatment with NaBH<sub>4</sub> gave benzylamine 18. A rigidified 2'-benzamide analogue 19 was synthesized via reaction of methyl 2-bromomethyl benzoate with amine 8. Finally, 8 was also subjected to diazotransfer to give curtailed azide 20. Removal of the acetonide in 35% TFA in water gave final products 21-25 (Scheme 3).

Scheme 3. Synthesis of 21–25 with Alternative Nitrogen-Based Linkers<sup>a</sup>



"Reagents and conditions. (a) PhSO<sub>2</sub>Cl, Et<sub>3</sub>N, CH<sub>2</sub>Cl<sub>2</sub>, 0 °C (73%); (b) PhNCO, pyr (86%); (c) [i] PhCHO, MeOH, mol. sieves, [ii] NaBH<sub>4</sub> (65%); (d) Methyl 2-(bromomethyl)benzoate, Et<sub>3</sub>N, MeOH, reflux (62%); (e) TfN<sub>3</sub>, MeOH, Et<sub>3</sub>N, CuSO<sub>4</sub> (82%); (f) 35% TFA in H<sub>2</sub>O (81–96%).

In line with what we observed for acetamide **10u**, truncated derivatives **15** and **25** also show promising potentiating activity in mature biofilms (EC<sub>50</sub> of 4.99 and 2.34  $\mu$ M, respectively, Table 1). However, they lose activity in the pretreatment setup when compared to lead compound **2**. Substitution of the 2'-amide in **2** with a sulfonamide or a urea linker is less tolerated. Benzylamine **23**, however, is performing well in the combination treatment regimen. Rigidification of **2** in the form of isoindolinone **24** causes a drop in activity.

It is clear from previous reports that ortho-substitution of the 5-phenyl ring (as in 2, Figure 1) increases activity, probably by distortion of the benzamide system into a nonplanar conformation.<sup>11,12</sup> However, the more polar phthalimide 3 and pyridazine 4 (Figure 1) showed remarkably good activity. Also biphenyl derivative 5 performed well as potentiator for VAN in S. aureus. Nine analogues were synthesized by combining alternative 5-amide substituents (predominantly chlorinated pyridines, next to nitrogen-containing 5-rings) with a fixed 2'-benzamide substituent. A selection is shown in Table 2 (33e-i). However, it was decided to modify both the 2'- and 5-position in a "mix and match" approach. A matrix of compounds with permutations of the different substituents was made (selection given in Table 2, i.e., 33j-y). This 33 series of analogues was synthesized in a manner that is essentially the same as described previously (Scheme S1 in Supporting Information).<sup>11</sup> A full overview of all mix and match compounds is given in Figure S2 and Table S2 in the Supporting Information.

From Table 2 it is clear that there is no additive effect in combining the properties on one side of the molecule, with some of the hybrid analogues showing no potentiating activity at all (e.g., 33e-g). The permutation approach, however, afforded several active potentiators. Among these analogues, compound 33k, which combines a 2'-ortho-chlorobenzamide and a 5-phthalimide moiety, emerged as the strongest potentiator in both pretreatment (EC<sub>50</sub> = 0.26  $\mu$ M) and

Compound <sup>a</sup>			EC50 (μM)	
		R <sub>2</sub>	Pretreatment	Combination treatment
HAM, 1	-	-	146	165
2		D H H H H	0.39	7.98
33e		P C C C C C C C C C C C C C C C C C C C	>250	232
33f		S H S H S H S H S H S H S H S H S H S H	>250	>250
33g		S H S H S H S H S H S H S H S H S H S H	200	>250
33h		D H H	29.3	23.5
33i		N S S S S S S S S S S S S S S S S S S S	46.7	58.2
33j	N <sup>3</sup> <sup>4</sup>	St E C	5.58	15.0
33k		PER C	0.26	1.27
330			5.85	11.4
33t	N N H	<sup>≯</sup> NH Ph	8.54	45.4
33w	Ph N <sup>2</sup>		24.7	12.1
33y			2.63	39.0

Table 2. Microbiological Evaluation of HAM Analogues from the Mix and Match Approach

<sup>*a*</sup>Compounds are in addition to VAN.

combination treatment (EC<sub>50</sub> = 1.27  $\mu$ M) regimens. In human liver microsomal fractions, this highly potent HAM derivative is not susceptible to enzymatic degradation by UGT enzymes. It is metabolized only to a small extent through Phase I metabolism, and it was found to be stable in human plasma at 37 °C (Tables S3 and S4 in Supporting Information). Promising potentiating activities were observed for several compounds of the 33 series in S. aureus Mu50, a highly resistant and representative clinical isolate. We confirmed (as for compound 2 in our previous publication<sup>11</sup>) that this effect is not restricted to only one isolate (Figure S3 in the Supporting Information). Representative HAM derivatives 33k and 33o did not have a direct inhibiting or dispersing effect on biofilms by themselves (Figure S4), nor did combinations of 33k or 33o with VAN reduce the MIC of the antibiotic (Figure S5), indicating a specific antibiotic potentiating effect in S. aureus biofilms.

In conclusion, we have successfully developed several active small molecule potentiators for vancomycin in *S. aureus*. Most

compounds with variation at the 2'-side of the molecule were devoid of activity. Exceptions are the truncated derivatives **10u**, **15**, and **25**, which demonstrate promising activities in combination therapy. A mix and match approach of favorable substituents led to the identification of a new lead compound **33k**, which shows exceptionally good activity in both *in vitro* tests and warrants further study.

### ASSOCIATED CONTENT

## **Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchem-lett.6b00315.

Experimental details and characterization data for the reported compounds, NMR spectra, and biological data (PDF)

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#### **Author Contributions**

All authors have given approval to the final version of the manuscript.

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# Notes

The authors declare no competing financial interest.

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#### ABBREVIATIONS

AMR, antimicrobial resistance; DIPEA, *N*,*N*-diisopropylethylamine; EDC, *N*-(3-(dimethylamino)propyl)-*N'*-ethylcarbodiimide; HAM, hamamelitannin; HOBt, 1-hydroxybenzotriazole; MIC, minimum inhibitory concentration; MRSA, methicillinresistant *Staphylococcus aureus*; QSI, quorum sensing inhibitor; TFA, trifluoroacetic acid; TraP, target of RNAIII activating protein; UGT, uridine 5'-diphospho-glucuronosyltransferase; VAN, vancomycin

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