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Hamamelitannin Analogues that Modulate Quorum Sensing as Potentiators of Antibiotics against Staphylococcus aureus. — Source link

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Topics: Quorum sensing, Antimicrobial and Potentiator

Hamamelitannin analogues that modulate quorum sensing as potentiators of antibiotics against **Staphylococcus aureus**

Don't Let the "Bad Bugs" Bite: Quorum Sensing Modulating Hamamelitannin Analogues as Potentiators of Antibiotics against *Staphylococcus aureus*

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Abstract: *Divide et impera*! Modulation of bacterial communication to potentiate the effect of existing antimicrobial drugs is a promising alternative to the development of novel antibiotics. In the present study, we synthesized 58 analogues of hamamelitannin (HAM, **1**), a quorum sensing inhibitor and antimicrobial potentiator. These efforts resulted in the identification of compound **38**, which increased the susceptibility of *Staphylococcus aureus* towards antibiotics both *in vitro* as well as in a *Caenorhabditis elegans* and in a mouse mammary gland infection model, while lacking cytotoxicity.

Antimicrobial resistance is a global public health challenge and new antimicrobial drugs are scarce.[1], [2] The chronic misuse and overuse of antibiotics, together with the fact that they impose selective pressure on bacteria, has contributed to the development of multiresistant pathogens. Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of these 'superbugs'.[3] *Staphylococcus aureus* (*S. aureus*) is a human commensal, but may turn into a versatile and dangerous pathogen capable of causing a range of infections, including skin and soft tissue infection, bacteremia, endocarditis and toxin-mediated disease, both in human and animals.[4], [5] Moreover, it is a leading cause of nosocomial infections.[6] *S. aureus* often resides within biofilms at the infection site, in which it displays increased resistance towards various forms of stress (e.g. antibiotic treatment, disinfectants, immune response).[7]

Potentiating the effect of existing antimicrobials and/or altering the virulence of pathogens may provide promising alternatives to combat biofilm-related infections.[8] Bacterial virulence is often mediated via quorum sensing (QS), a cell-to-cell communication system by which bacteria sense population density and control genetically mediated responses. *S. aureus* strains use at least two different QS systems to regulate their virulence: the *agr* system and the RAP/TRAP system.[9] Both systems are reported to alter gene expression through the control of RNAIII. The use of QS modulators as potentiators of antibiotics has been proposed in literature.[8], [10], [11], [12]

Recently, hamamelitannin (HAM [**1**], Figure 1), a natural product isolated from the American witch-hazel (*Hamamelis virginiana*), was shown to potentiate the effect of antimicrobials against *S. aureus* by interfering with QS. [13], [14], [15] From a medicinal chemistry perspective, the structure of HAM shows important liabilities. It consists of a flexible central D-hamamelose scaffold, whose primary hydroxyl groups are esterified with gallic acid (Figure 1). The many hydroxyl functions make HAM very polar, which might compromise its bioavailability. The aromatic hydroxyl functions make the molecule prone to oxidation and glucuronidation. Moreover, it is doubtful whether the ester linkers will be metabolically stable *in vivo*.

These liabilities stimulated us to investigate three HAM modifications: replacement of the ester groups with isosteric linker moieties, systematic modification or elimination of the aromatic hydroxyl groups, and finally removal of the anomeric hydroxyl group, thereby generating a rigid and structurally well-defined tetrahydrofuran core.

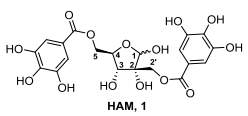

Figure 1. Structure of hamamelitannin (HAM, **1**).

The branched azidolactol **2** was considered a convenient starting material for our purposes (Scheme 1).[16] A three-step sequence gave the two readily separable anomers **4a** and **4b** in approximately equal amounts. For convenience of analysis, it was decided to utilize the more apolar β-anomer **4b** for the generation of the bis-azide precursor **5**. Staudinger reduction, followed by EDC-mediated acylation of the resulting diamine **6** and final deprotection, gave the desired HAM analogues **8f-l**.

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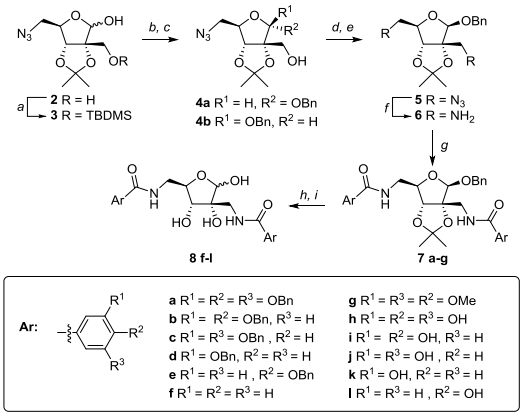


Scheme 1. Synthesis of compounds **8f-l** from **2**. Reagents and conditions (yields in parentheses): a) TBDMSCl, imidazole, DMF, 0 °C, 16h (63%); b) BnBr, NaH, DMF, 0 °C, 16h; c) TBAF, THF, rt, 4h (**4a** 41.8%, **4b** 42.5%); d) based on compound **4b**: MsCl, Et_3N, DCM, rt, 2h (95%); e) NaN_3, DMF, 90 °C, 48h (70%); f) PMe_3, THF, H_2O; g) $ArCO_2H$, EDC.HCl, DMAP, HOBt, DMF, rt, 16h; h) H_2, Pd/C, HOAc, 5h; i) 35% TFA, H_2O, rt, 3h.

The strategy to remove the anomeric hydroxyl is shown in Scheme 2. Intermediate **2** was reduced to triol **9** and cyclized to generate tosylate **10**.[17] The rigidity of the dioxolane ring system ensures that only the *cis*-bicycle is formed. Tosylate **10** was used to generate a series of 51 amide analogues of HAM, featuring either two identical (**14b**, **14c**) or two different acyl groups (**18-66**), a selection of which is given in Scheme 2. A complete overview of the designed HAM-analogues is given in Figure S1.

Crystals structures of compounds **18**, **19**, **38** and **50** allowed unambiguous stereochemical assignment (Figure S2-S5).

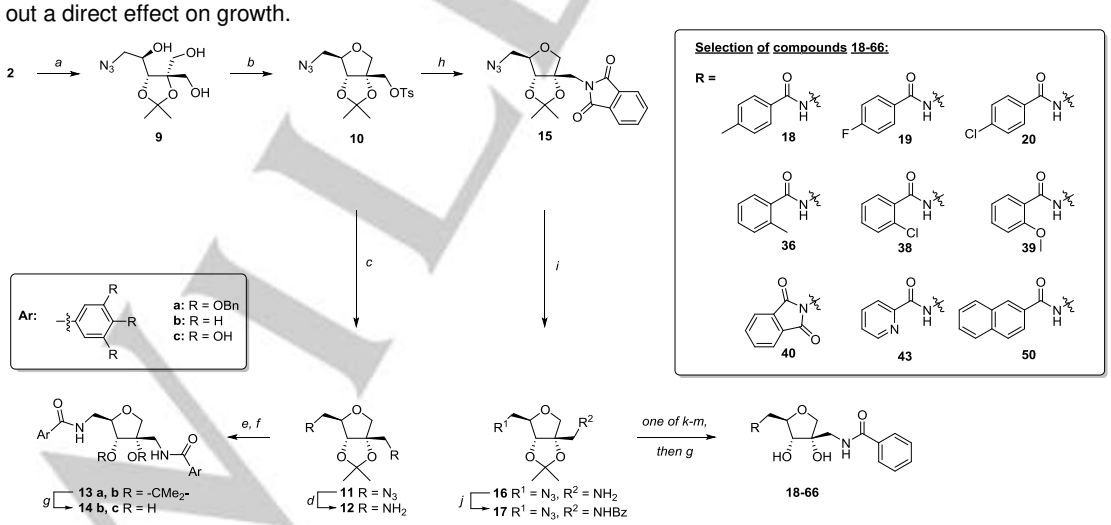
The minimum inhibitory concentrations (MIC) of all final compounds against *S. aureus* Mu50 were higher than 500 µM, which rules out a direct effect on growth.

Table 1. The concentration of HAM analogue needed to double the effect of the antibiotic *in vitro* under pre-treatment or combination treatment regimens as measured by the number of surviving *S. aureus* Mu50 biofilm cells (expressed as EC_{50} values).

Compound	EC_{50} (µM)	
	Pretreatment	Combination treatment
1, HAM	145.5	165.1
8f	73.63	154.2
8h	63.82	191.1
14b	96.97	93.55
14c	77.12	146.9
20	18.01	21.39
36	13.67	82.62
38	0.389	7.976
39	53.21	65.12
40	5.013	8.298
43	70.42	104.4

Subsequently, HAM and analogues were tested for their ability to augment biofilm susceptibility to vancomycin (VAN) both under pretreatment and under combination treatment regimens. When comparing HAM with compound **8h**, it can be concluded that substitution of esters by amides is well-tolerated (Table 1 and S1). Elimination of the phenolic groups does not lead to a significant reduction in activity, while removal of the anomeric hydroxyl group is also well-tolerated (cf. pairs **8f**/**14b** and **8h**/**14c**).

Figure 2 summarizes the *in vitro* results for the most potent HAM analogue (**38**). When used alone, VAN (20 µg/ml) resulted only in a minor reduction of the number of *S. aureus* sessile cells (approximately 30%). In contrast, combined treatment of VAN with HAM or **38** resulted in significantly more killing of *S. aureus* Mu50 biofilm cells, both under pretreatment as under combination treatment regimens (Figure 2).



Scheme 2. Synthesis of analogues **14b,c** and **18-66** from intermediate **10**. Reagents and conditions (yields in parentheses): $NaBH_4$, MeOH, 0 °C, 16h (93%); b) [i] TsCl, pyridine, rt, 3h, [ii] 60 °C, 16h (72%); c) NaN_3, DMF, 80 °C, 16h (86%); d) PMe_3, THF, H_2O; e) $ArCO_2H$, EDC.HCl, DMAP, HOBt, DMF, rt, 16h; f) H_2, Pd/C, MeOH, 2h; g) 35% TFA, H_2O, rt, 3h; h) K phthalimide, NaI, DMF, 90 °C, 16h (86%); i) $N_2H_4.H_2O$, EtOH, reflux, 4 h (93%); j) BzCl, Et_3N, DCM, 0 °C, 3 h (99%); k) [i] PMe_3, THF, H_2O, [ii] RCO_2H, EDC.HCl, DIPEA, HOBt, DMF, rt, 16h; l) [i] PMe_3, THF, H_2O, [ii] $(AcO)_2O$, DIPEA, 0 °C, 16h; m) [i] PMe_3, THF, H_2O, [ii] $(CH_3)_3CCOCl$, Et_3N, 0 °C, 16h.

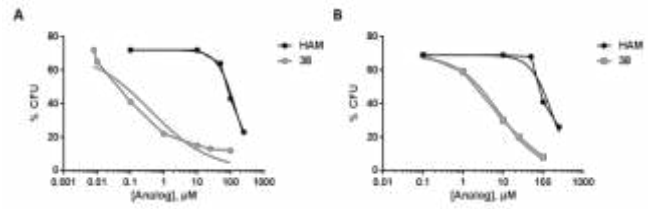


Figure 2. The percentage CFU/biofilm ± s.d. (compared to 100% untreated control biofilm) for 24 h biofilms that were pretreated i.e. **A**) formed in the presence of different concentrations of either HAM or **38** and subsequently treated with vancomycin (VAN; 20 µg/ml) or **B**) treated with a combination of VAN (20 µg/ml) and either HAM or **38** (both used at different concentrations).

In pretreatment conditions, **38** has an EC_{50} of 0.389 µM (Table 1), which is an approximate 400-fold improvement compared to HAM (EC_{50} = 145.5 µM).

The *ortho*-chloro derivative **38** shows a much better pretreatment activity than its *para*-chloro congener **20** (EC_{50} = 18.01 µM). The increased activity upon *ortho* substitution of the 5-benzamide moiety (also observed with *ortho*-methyl analogue **36**) could result from a twist of the phenyl ring from the C_5-N(H)-C(O)-C_{ipso} plane, allegedly improving interaction with the target. This might also explain why compounds **39** and **43** lose activity due to the hydrogen-bonded pseudoring formation. Derivatives with sterically demanding (i.e. indoles, naphthalenes, disubstituted benzamides) and longer, more flexible amide substituents show reduced activity, which is potentially linked to confounding steric effects (Table S1-S2). Non-aromatic amides also demonstrated poor activity. Initial efforts to optimize the activity of **38** by varying its "eastern" benzamide moiety proved more challenging (results not shown).

Compound **38** was selected for further study owing to its very promising activity and improved "druglikeness". *In vitro* evaluation of its metabolic stability shows that **38** is stable in human plasma after incubation for 24h at 37 °C (Table S3). Moreover, it is stable in the presence of rat and human S9 microsomal fractions indicating no Phase-I or -II metabolism (Table S4). Finally, compound **38** displayed no cytotoxicity in MRC-5 lung fibroblast cells at 128 µM, suggesting acceptable therapeutic windows (Table S5).

We recently showed that HAM increases susceptibility of *S. aureus* towards a wide range of antibiotics by affecting peptidoglycan thickness and eDNA release through the QS receptor TraP.[15] However, it was unclear at this point whether this would still be true for the synthetic HAM analogue **38**, originating from phenotypical optimization.

Firstly, we investigated the spectrum of **38**. Although this analogue had no effect on the MIC of different antibiotics at 100 µM (Table S6), it increased the susceptibility of *S. aureus* biofilm cells towards different classes of antibiotics (Figure 3A), indicating that its effect is not limited to a combination with VAN.

Secondly, we addressed the specificity of **38** for the TraP receptor in the *S. aureus* QS system by evaluating the biofilm susceptibility of a *S. aureus* ATCC 49230 wildtype (WT), a Δ*traP* mutant and a Δ*traP pLI50-U1 traP* complemented strain against VAN when used alone or in combination with **38** (100 µM). Although the WT strain became more susceptible when VAN was used in combination with **38**, this increased susceptibility to VAN disappeared in *S. aureus ΔtraP* (Figure 3B). The complemented Δ*traP* mutant strain displayed decreased susceptibility towards VAN alone, while its susceptibility was increased when VAN was used in combination with **38**. These observations strongly indicate that the effect of **38** on *S. aureus* biofilm susceptibility involves the TraP receptor. Figure 3C shows that **38** increased lysostaphin susceptibility, indicating changes in peptidoglycan structure. Quantification of eDNA in *S. aureus* ATCC 49230 biofilms showed that less eDNA was present in biofilms of the WT strain treated with **38**, compared to control (Figure 3D). Although significantly less eDNA was present in the biofilm matrix of the Δ*traP* mutant strain compared to the WT strain, no difference in the amount of eDNA was observed in biofilms of these mutants formed in the absence versus the presence of **38** (Figure 3D). In contrast, increased eDNA production was seen for the complemented strain, compared to the Δ*traP* mutant when no **38** was used and eDNA production significantly decreased when **38** was present during biofilm formation. These results indicate that **38** alters biofilm susceptibility by affecting both peptidoglycan structure and eDNA release by interfering with the TraP receptor, which is in line with what we previously observed for HAM.[15]

Finally, we evaluated the effect of **38** on *S. aureus* susceptibility in a *Caenorhabditis elegans* (*C. elegans*) infection model and in a mouse mammary gland infection model. Although treatment with **38** and VAN alone resulted in an increased survival of infected *C. elegans* nematodes (Table S7), either **38**, or HAM or VAN alone had no effect on the number of bacteria present in *C. elegans* nematodes after infection (Figure 4A). In contrast, significantly less CFU/nematode were present upon combined treatment of either **38** or HAM with VAN compared to treatment with VAN alone (Figure 4A). The effect of **38** was stronger than that of HAM.

In a mouse model of *S. aureus* mastitis, either **38** or HAM had no effect on the number of CFU present in the infected mouse mammary glands (Figure 4B). Significantly less CFU/g mammary gland were present when CFL was combined with **38** or HAM (Figure 4B). Again, the effect of **38** was stronger than that of HAM. Macroscopic signs of inflammation were mainly observed in the glands of mice receiving no treatment and to a much lesser extent in the glands of mice receiving a treatment with **38** and/or CFL (Figure S6). In addition, an influx of neutrophils was observed in the alveoli of the glands in the untreated conditions or the glands treated with **38** or HAM alone (Figure S7). However, this innate immune response was not observed in the mice receiving treatment with CFL or a combination of CFL and **38** or HAM.

In conclusion, we developed a practical synthetic route for the preparation of a library of 58 HAM analogues. Several compounds show promising activity on biofilm susceptibility. The 5-*ortho*-chlorobenzamide derivative **38** emerged as the strongest potentiator. Data from two complementary *in vivo* infection models suggest that **38** has the potential to increase the effect of antibiotics *in vivo* and that this effect is superior to that of HAM. Staphylococcal resistance to antibiotics is a growing public health threat of broad concern and we believe that the small-molecule potentiators described herein can contribute in the fight against chronic and difficult-to-treat infections.

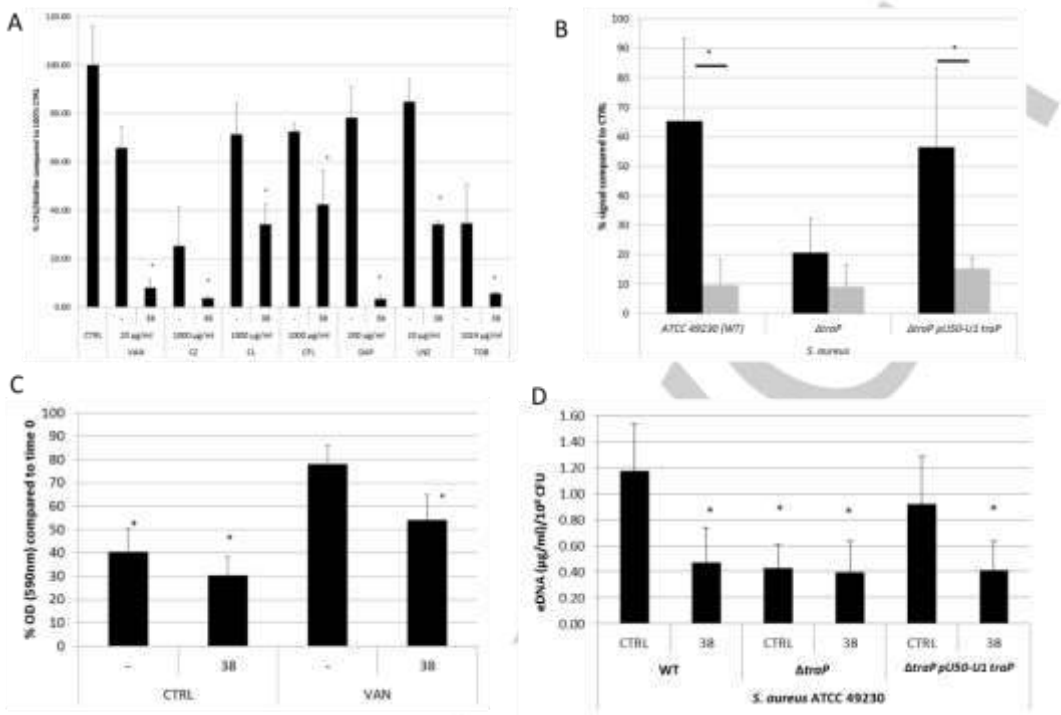


Figure 3. Effect of treatment on *in vitro* susceptibility of *S. aureus* biofilms. **A)** The percentage CFU/biofilm ± s.d. (compared to untreated control biofilm) for *S. aureus* Mu50 biofilm cells exposed to vancomycin (VAN), cefazolin (CZ), cefalonium (CL), cephalexin (CFL), daptomycin (DAP), linezolid (LNZ) or tobramycin (TOB) alone or in combination with **38**. *: significantly increased killing for the combination of the antibiotic and **38** compared to treatment with the antibiotic alone ($n \geq 9$; one-way; $p < 0.05$). **B)** Biofilms of *S. aureus* ATCC 49230 (WT), Δ*traP* mutant and the *traP* complemented strain *ΔtraP pLI50-U1 traP* were exposed to VAN alone (black bars) or a combination of **38** and VAN (grey bars). Cell viability was quantified by CTB staining and signals are presented as percentages (average ± s.d.) compared to the signal of an untreated biofilm. *: significantly different signals were observed between both treatments ($n \geq 9$; one-way; $p < 0.01$). **C)** The OD590 nm (average ± s.d.) after 10 min lysostaphin treatment of *S. aureus* Mu50 biofilm cells receiving either no pre-treatment (CTRL) or a pre-treatment with **38**, VAN or a combination of **38** and VAN. The OD590nm after 10min was compared to the OD590 before the addition of lysostaphin (set at 100 %).*: the percentage OD590 nm is significantly different from that of the cells receiving pre-treatment with VAN ($n \geq 6$; one-way; $p < 0.05$). **D)** The amount of eDNA (average ± s.d.) present in biofilms of WT *S. aureus* ATCC 49230, Δ*traP* mutant and the *traP* complemented strain Δ*traP pLI50-U1 traP* formed in the absence (CTRL) or presence of **38**. *: indicates significant ($p < 0.05$) differences between the amount of eDNA present in the treated biofilms compared to the untreated WT biofilm.

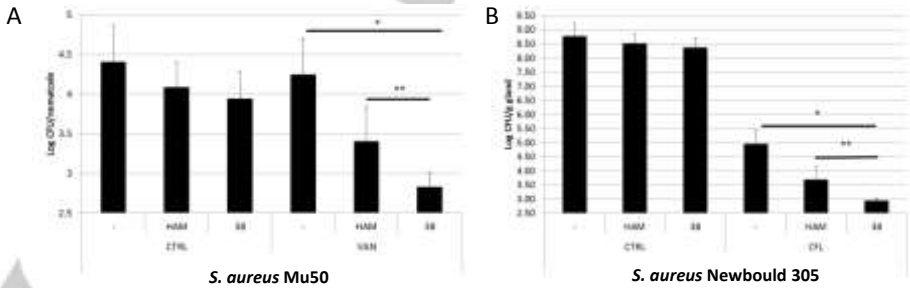


Figure 4. Effect of treatment on *in vivo* susceptibility of *S. aureus*. **A)** Log CFU/nematode (average ± s.d.) in *C. elegans* nematodes infected with *S. aureus* Mu50 biofilm cells, receiving no treatment (CTRL) or a treatment with HAM, **38** or VAN or a combination of VAN and either HAM or **38**. **B)** Log CFU/g mammary gland (average ± s.d.) of mice infected with *S. aureus* Newbould305 receiving no treatment or a intramammary treatment with HAM, **38** or cephalexin (CFL) alone or a combination of CFL and either HAM or **38**. *: significant differences in log CFU/nematode (A) or log CFU/g mammary gland (B) between combination treatment and treatment with the antibiotic alone ($p < 0.01$). **: significant differences in log CFU/nematode (A) or log CFU/g mammary gland (B) between combination treatment with antibiotic and **38** compared to combination treatment with the antibiotic and HAM ($p < 0.01$).

Experimental Section

Experimental details can be found in the supporting information.

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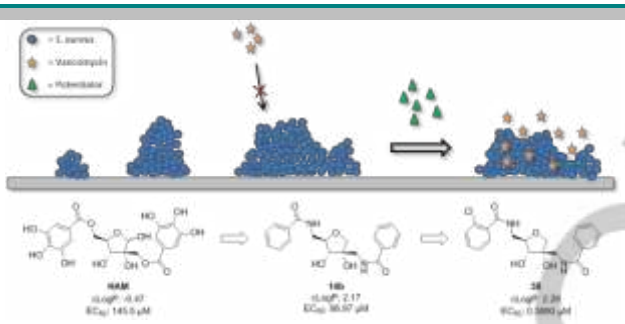
COMMUNICATION

New potentiator drugs for superbugs! *S. aureus* is notorious for its resistant, biofilm-related infections. The discovery of hamamelitannin as an antimicrobial potentiator, led us to investigate the SAR. We identified **38** as a potent and metabolically stable analogue, which can contribute in the ever-lasting fight between humanity and micro-organism.



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