Biofilms

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# Hamamelitannin Analogues that Modulate Quorum Sensing as Potentiators of Antibiotics against *Staphylococcus aureus*

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***Abstract:*** *The 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), a quorum sensing inhibitor and antimicrobial potentiator. These efforts resulted in the identification of an analogue that increases the susceptibility of Staphylococcus aureus towards antibiotics in vitro, in Caenorhabditis elegans, and in a mouse mammary gland infection model, without showing 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 multidrug-resistant pathogens. Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of these "superbugs".[3] *Staphylococcus aureus* (*S. aureus*) is a human commensal microorganism but it can 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, in both humans and other 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, for example, antibiotic treatment, disinfectants, and the immune response.[7]

Potentiating the effect of existing antimicrobial agents and/or altering the virulence of pathogens may provide promising approaches to combating biofilm-related infections.[8] Bacterial virulence is often mediated by 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 have been reported to alter gene expression through the control of RNAIII, a small mRNA that is known to regulate many *S. aureus* genes. The use of QS modulators as potentiators of antibiotics has been proposed.[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, the primary hydroxy groups of which are esterified with gallic acid (Figure 1). The high number of hydroxy functions make HAM very polar, which might compromise its bioavailability. The aromatic hydroxy functions make the molecule prone to oxidation and glucuronidation. Moreover, it is doubtful whether the ester linkers will be metabolically stable in vivo.

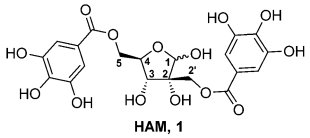


***Figure 1.*** Structure of hamamelitannin (HAM, **1**).

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 hydroxy groups, and finally removal of the anomeric hydroxy group, thereby generating a rigid and structurally well-defined tetrahydrofuran core.

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

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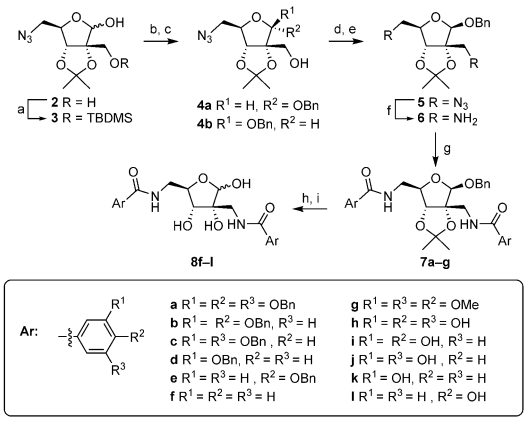


***Scheme 1.*** Synthesis of compounds **8 f–l** from **2**. Reagents and conditions (yields in parentheses): a) TBDMSCl, imidazole, DMF, 0 °C, 16 h (63 %); b) BnBr, NaH, DMF, 0 °C, 16 h; c) TBAF, THF, RT, 4 h (**4 a** 41.8 %, **4 b** 42.5 %); d) based on compound **4 b**: MsCl, $Et_3N$, DCM, RT, 2 h (95 %); e) $NaN_3$, DMF, 90 °C, 48 h (70 %); f) $PMe_3$, THF, $H_2O$; g) $ArCO_2H$, EDC·HCl, DMAP, HOBt, DMF, RT, 16 h; h) $H_2$, Pd/C, HOAc, 5 h; i) 35 % TFA, $H_2O$, RT, 3 h. TBDMSCl = *tert*-butyldimethylsilyl chloride, DMF = *N*,*N*-dimethylformamide, Bn = benzyl, TBAF = tetra-*n*-butylammonium fluoride, THF = tetrahydrofuran, Ms = methanesulfonyl, EDC.HCl = 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, DMAP = 4-dimethylaminopyridine, HOBt = 1-hydroxybenzotriazole.

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 **8 f–l**.

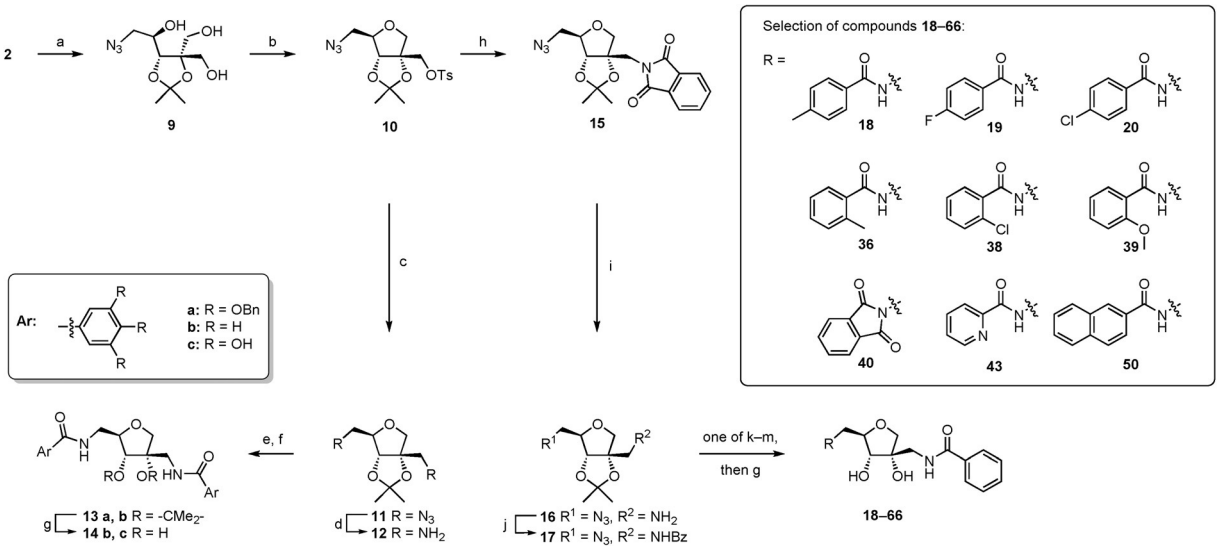
The strategy to remove the anomeric hydroxy group is shown in Scheme 2. Intermediate **2** was reduced to triol **9** and cyclized to generate tosylate **10**.[17] The rigidity of the dioxolane ring system ensures that only the *cis*-bicycle is formed. Tosylate **10** was used to generate a series of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

Crystal structures of compounds **18**, **19**, **38**, and **50** allowed unambiguous stereochemical assignment (Figures S2–S5). The minimum inhibitory concentration (MIC) values of all of the final compounds against *S. aureus* Mu50 were higher than 500 μM, which rules out a direct effect on growth.

Subsequently, HAM and the HAM 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 **8 h**, it can be concluded that the substitution of esters with amides is well tolerated (Table 1 and Table S1). Elimination of the phenolic groups does not lead to a significant reduction in activity, while removal of the anomeric hydroxy group is also well tolerated (cf. pairs **8 f**/**14 b** and **8 h**/**14 c**).

Figure 2 summarizes the in vitro results for the most potent HAM analogue (**38**). When used alone, VAN (20 $\mu g\,mL^{-1}$) resulted only in a minor reduction of the number of *S. aureus* sessile cells (approximately 30 %). By contrast, combined treatment of VAN with HAM or **38** resulted in significantly more killing of *S. aureus* Mu50 biofilm cells under both pretreatment and combination treatment regimens (Figure 2).

Under pretreatment conditions, the concentration of HAM analogue **38** needed to double the effect of antibiotic ($EC_{50}$) is 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** also shows a much better pretreat-



***Scheme 2.*** Synthesis of analogues **14 b,c** and **18–66** from intermediate **10**. Reagents and conditions (yields in parentheses): $NaBH_4$, MeOH, 0 °C, 16 h (93 %); b) i. TsCl, pyridine, RT, 3 h, ii. 60 °C, 16 h (72 %); c) $NaN_3$, DMF, 80 °C, 16 h (86 %); d) $PMe_3$, THF, $H_2O$; e) $ArCO_2H$, EDC.HCl, DMAP, HOBt, DMF, RT, 16 h; f) $H_2$, Pd/C, MeOH, 2 h; g) 35 % TFA, $H_2O$, RT, 3 h; h) K phthalimide, NaI, DMF, 90 °C, 16 h (86 %); i) $N_2H_4{\cdot}H_2O$, 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, 16 h; l) i. $PMe_3$, THF, $H_2O$, ii. $(AcO)_2O$, DIPEA, 0 °C, 16 h; m) i. $PMe_3$, THF, $H_2O$, ii. $(CH_3)_3CCOCl$, $Et_3N$, 0 °C, 16 h. TFA = trifluoroacetic acid, Bz = benzoyl, DIPEA = *N*,*N*-diisopropylethylamine.

***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] | |
|---|---|---|
| | Pre-treatment | 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 |

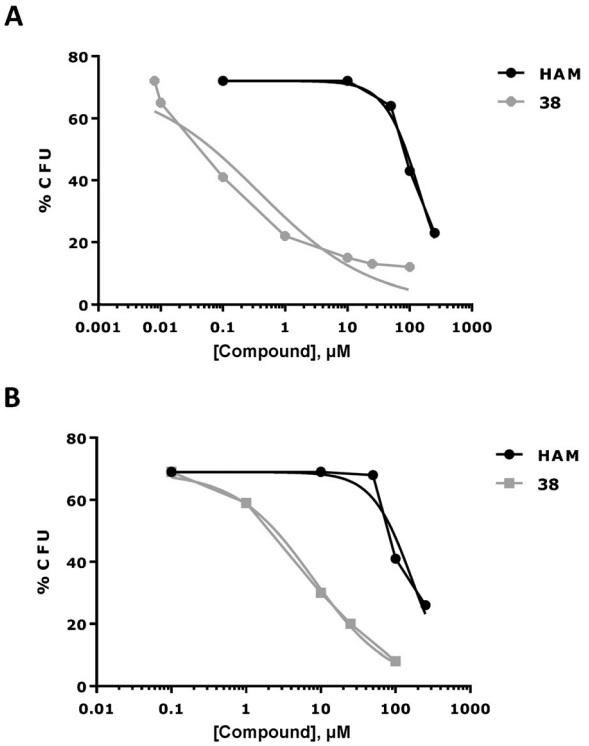



***Figure 2.*** The percentage colony-forming units (CFU)/biofilm ± SD (i.e., per well in a 96 well plate; compared to 100% untreated control biofilm) for 24 h biofilms A) formed in the presence of different concentrations of either HAM or **38** and subsequently treated with vancomycin (VAN; 20 μg mL$^{-1}$) or B) treated with a combination of VAN (20 μg mL$^{-1}$) and either HAM or **38** (both used at different concentrations).

ment 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 twisting of the phenyl ring from the $C_5$-N(H)-C(O)-$C_{ipso}$ plane, thereby improving interaction with the target. This might also explain why compounds **39** and **43** lose activity as a result of hydrogen-bonded pseudoring formation. Derivatives with sterically demanding (e.g., indoles, naphthalenes, disubstituted benzamides) and longer, more flexible amide substituents show reduced activity, which is potentially linked to confounding steric effects (Tables 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 "drug-like" properties. In vitro evaluation of its metabolic stability showed that **38** is stable in human plasma after incubation for 24 h at 37 °C (Table S3). Moreover, it is stable in the presence of rat and human S9 microsomal fractions, thus indicating no Phase-I or -II metabolism (Table S4). Finally, compound **38** displayed no cytotoxicity in MRC-5 lung fibroblast cells at 128 μM, thus suggesting an acceptable therapeutic window (Table S5).

We recently showed that HAM increases the susceptibility of *S. aureus* towards a wide range of antibiotics by affecting peptidoglycan thickness and extracellular DNA (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**, which originated from phenotypic optimization (i.e., in terms of potentiator activity).

First, we investigated the activity 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), thus indicating that its effect is not limited to combination with VAN.

Second, we addressed the specificity of **38** for the TraP receptor in the *S. aureus* QS system by evaluating the biofilm susceptibility of *S. aureus* ATCC 49230 wildtype (WT), a Δ*traP* mutant, and a Δ*traP pLI50-U1 traP*-complemented strain to 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, thus 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 the 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). By 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 to antibiotic treatment by affecting both peptidoglycan structure and eDNA release through interfering with the TraP receptor, which is in line with what we previously observed for HAM.[15]

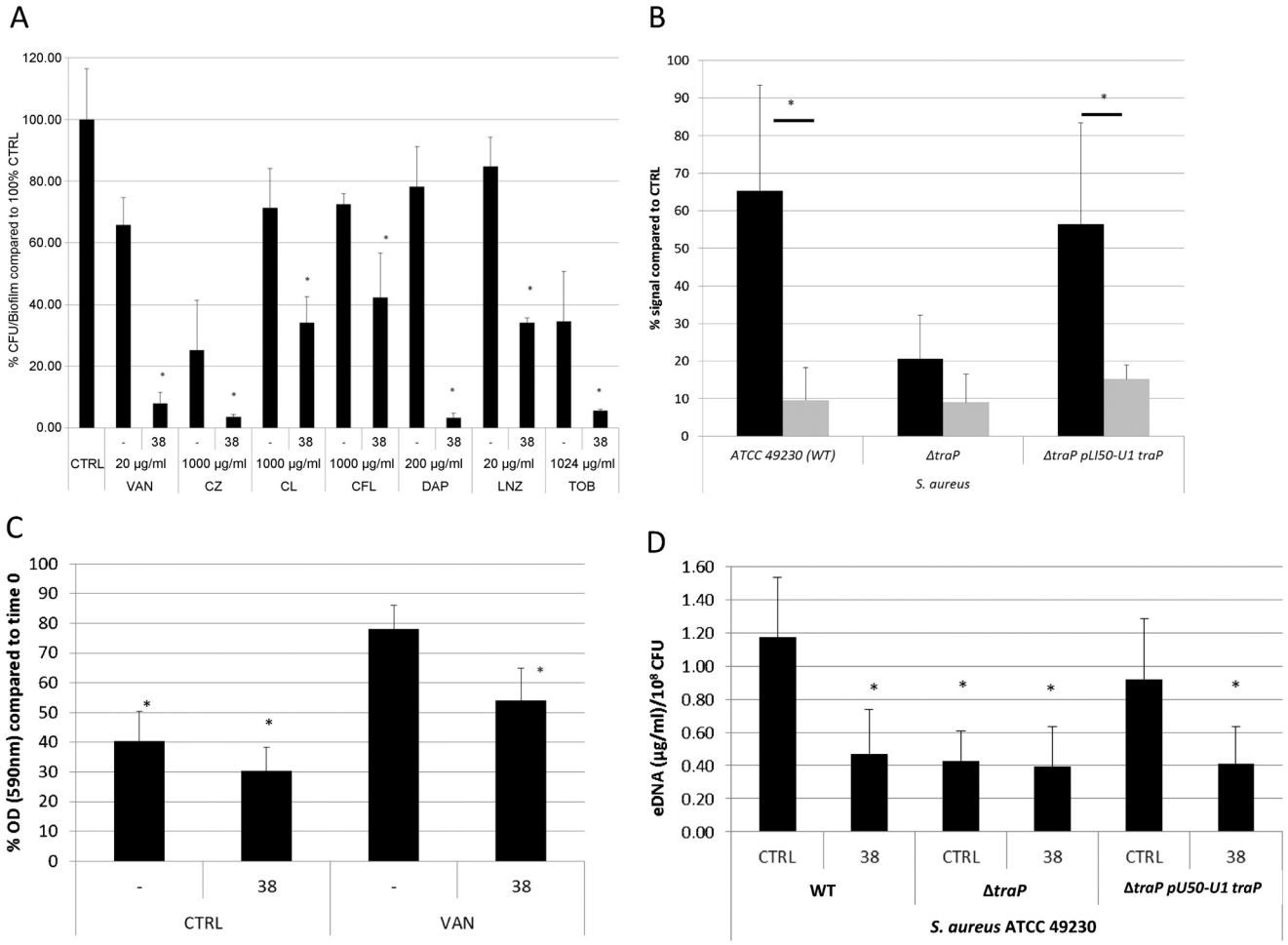



***Figure 3.*** Effect of treatment on the in vitro susceptibility of *S. aureus* biofilms to antibiotic treatment. A) The percentage CFU/biofilm ± SD (compared to untreated control biofilm) for *S. aureus* Mu50 biofilm cells exposed to vancomycin (VAN), cefazolin (CZ), cefalonium (CL), cephalexin (CFL), 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 ANOVA; $p < 0.05$). B) Biofilms of *S. aureus* ATCC 49230 (WT), a 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 (mean ± SD) compared to the signal of an untreated biofilm. *: significantly different signals were observed between the two treatments ($n \geq 9$; one-way ANOVA; $p < 0.01$). C) The OD590 nm (mean ± SD) after 10 min lysostaphin treatment of *S. aureus* Mu50 biofilm cells receiving either no pre-treatment (CTRL) or pre-treatment with **38**, VAN, or a combination of **38** and VAN. The OD590nm after 10 min 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 ANOVA; $p < 0.05$). D) The amount of eDNA (mean ± SD) present in biofilms of WT *S. aureus* ATCC 49230, a 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.

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 increased survival of infected *C. elegans* nematodes (Table S7), either **38**, HAM, or VAN alone had no effect on the number of bacteria present in *C. elegans* nematodes after infection (Figure 4 A). By contrast, significantly fewer colony-forming units (CFU) per nematode were present upon combined treatment with VAN and either **38** or HAM compared to treatment with VAN alone (Figure 4 A), and 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 4 B). Significantly fewer CFU/g mammary gland were present when cephalexin (CFL) was combined with **38** or HAM (Figure 4 B). 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 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,

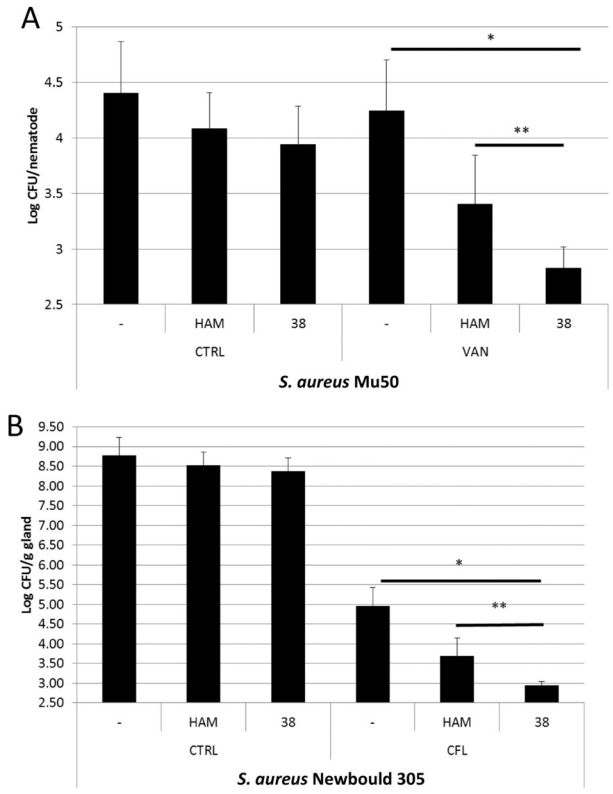



***Figure 4.*** Effect of treatment on the in vivo susceptibility of *S. aureus* to antibiotic treatment. A) Log CFU/nematode (mean ± SD) in *C. elegans* nematodes infected with *S. aureus* Mu50 biofilm cells, receiving no treatment (CTRL) or treatment with HAM, **38**, VAN, or a combination of VAN and either HAM or **38**. B) Log CFU/g mammary gland (mean ± SD) of mice infected with *S. aureus* Newbould305 receiving either no treatment or an 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 ($p < 0.01$).

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 in increasing biofilm susceptibility to antibiotic treatment. 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.

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