Tailored Phenyl Esters Inhibit ClpXP and Attenuate *Staphylococcus aureus* α-Hemolysin Secretion

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Novel strategies against multidrug-resistant bacteria are urgently needed in order to overcome the current silent pandemic. Manipulation of toxin production in pathogenic species serves as a promising approach to attenuate virulence and prevent infections. In many bacteria such as *Staphylococcus aureus* or *Listeria monocyotgenes*, serine protease ClpXP is a key contributor to virulence and thus represents a prime target for antimicrobial drug discovery. The limited stability of previous electrophilic warheads has prevented a sustained effect of virulence attenuation in bacterial culture. Here, we systemati-

cally tailor the stability and inhibitory potency of phenyl ester ClpXP inhibitors by steric shielding of the ester bond and fine-tuning the phenol leaving group. Out of 17 derivatives, two (MAS-19 and MAS-30) inhibited *S. aureus* ClpP peptidase and ClpXP protease activities by >60% at 1 μM. Furthermore, the novel inhibitors did not exhibit pronounced cytotoxicity against human and bacterial cells. Unlike the first generation phenyl-ester AV170, these molecules attenuated *S. aureus* virulence markedly and displayed increased stability in aqueous buffer compared to the previous benchmark AV170.

Introduction

With the rapid spread of multidrug-resistant bacteria, formerly easy-to-treat infections have progressively become a major health threat and led to increasing number of fatalities.[1] Given the absence of novel antimicrobial chemical entities, the limited scope of addressed targets and the propensity of bacteria to develop resistances due to extrinsic stress, novel strategies for antimicrobial development are urgently needed.[2] The anti-virulence approach focuses on the reduction of toxin release resulting in disarmed bacterial strains that are less infective and can be eliminated by the host immune response. Moreover, as there is less selective pressure on the bacteria, the need for resistance development is alleviated.[2c,d,3] Despite this intriguing perspective, this approach is still in its infancy.[2c] An important prerequisite for the anti-virulence strategy is the selection of appropriate targets which ideally control several toxins and thus have a major impact on pathogenicity. One such target is the bacterial protease ClpXP, which was previously shown to be an important switch for global virulence regulation in several pathogenic bacteria.[4] ClpXP is a serine protease composed of two heptameric ClpP peptidase units and one or two hexameric ClpX chaperones.[5] ClpX recognizes and unfolds proteins destined for digestion and threads the unfolded peptide chain

into the proteolytic chamber of ClpP through both apical sites for complete degradation.[6] After digest cleaved peptides are released through equatorial exit pores(Figure 1A).[7] ClpP activity can be chemically modulated in two different ways. Acyldepsipeptides (ADEPs) have been shown to increase ClpP activity resulting in the uncontrolled degradation of flexible and unfolded proteins,[8] thus making overactivation a promising strategy for antibiotic research by disturbing cell homeostasis.[8b] In contrast, chemical inhibition of ClpP revealed attenuation of virulence in *Staphylococcus aureus*.[10a] Although the exact mechanism of ClpP mediated virulence reduction is not fully elucidated, upregulation of DNA-binding protein repressor of toxins (Rot) was observed upon ClpP inhibition. Rot is important for regulation of virulence factors like α-hemolysin, which is essential for promotion of pathogenesis.[9] Inhibitors of both ClpP and ClpX have been discovered,[10] including beta-lactones[10a] and phenyl esters, which covalently bind the active site serine (Ser98) of ClpP.[8] The latter class was previously discovered via a high-throughput screen (HTS) of about 137,000 compounds against *S. aureus* ClpP. One of the most potent hits, AV170, bears a trimethoxyphenylethyl substituent next to the phenyl ester group, which proved critical for binding to the protease (Figure 1B).[11] Despite its potent inhibition of peptidase and protease activity, only limited reduction of *S. aureus* virulence was observed for AV170. Studies on the stability of AV170 revealed rapid hydrolysis of the unshielded ester moiety, which may result in lower cellular concentrations than needed for sustained inhibition of ClpXP in living bacteria.[11] Thus, a next-generation of ClpP inhibitors with enhanced resistance to hydrolysis of the ester bond is needed.

Notably, phenyl esters such as sivelestat and camostat (Figure 1C) are approved protease inhibitors for oral treatment of chronic pancreatitis and acute respiratory failure, respectively,[12] thus generation of stabilized phenyl ester ClpXP inhibitors should be feasible in principle. Inspired by these two drugs, we developed a new generation of ClpXP phenyl esters

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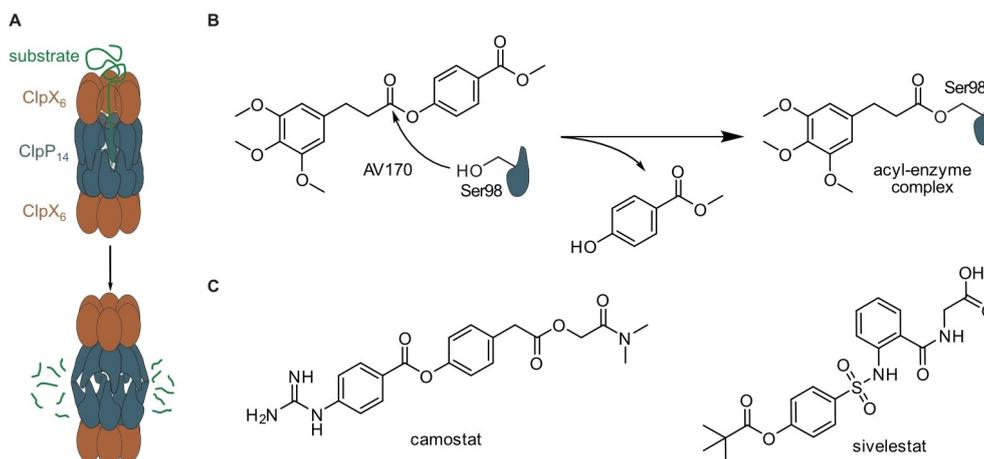**Europe**
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Figure 1. (A) Structure of the proteolytic ClpXP complex, consisting of tetradecameric, barrel-shaped ClpP$_{14}$ (blue) and one or two hexameric ClpX$_6$ ATPases (orange). ClpX$_6$ ATPases bind to the axial pores, recognize and unfold substrate proteins to thread them into the inner proteolytic chamber of ClpP. Cleaved peptides are released through equatorial exit pores. (B) Inhibitory mechanism of benchmark phenyl ester AV170 via transesterification with ClpP active site serine (Ser98). The resulting acyl–enzyme complex inactivates Ser98. (C) Chemical structures of phenyl ester drugs camostat and sivelestat.

with improved stability, retained ClpXP inhibitory potency and, contrary to the first generation, also pronounced anti-hemolytic activity.

Results

A hallmark of the camostat scaffold is the placement of a bulky phenyl substituent next to the ester bond, which could shield this moiety more effectively from hydrolysis. The stability of camostat is sufficient to be administered orally. Re-inspecting our results from the previous HTS revealed two related hit molecules bearing either a benzoic acid- (AV126) or a piperidyl- (AV127) scaffold (Figure 2). In fact, these molecules have been shown to have similar potency against ClpP and ClpXP as AV170,[11] yet, like camostat and sivelestat, have more sterically shielded ester bonds than AV170. In order to investigate the potential of these two scaffolds to serve as next-generation inhibitors with improved stability, we devised several new derivatives based on AV126 and AV127. For AV126 we performed systematic alterations on the benzoic acid-substituent to decipher both the effect of various electron-donating and withdrawing substitutions (MAS-14, MAS-15, MAS-16) and of differently configured sulfonyl groups (MAS-21, MAS-26, MAS-83). MAS-53, a simplified scaffold inspired by camostat was included as well (Figure 2). AV127 derivatives comprised various substitutions at the piperidyl amine (MAS-19, MAS-27, MAS-30), a replacement of the heterocycle by cyclohexane (MAS-55) as well as various leaving groups (MAS-33, MAS-50, MAS-66, MAS-72) (Figure 2, right). MAS-17 and MAS-20 were designed as simplified compounds lacking a phenyl or piperidyl moiety (Supporting Figure S1B).

All derivatives were synthesized in a modular way that coupled the respective acid and alcohol in a Steglich esterification using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 4-dimethyl–aminopyridine (DMAP) as coupling

reagents (Supporting Scheme S1). The products synthesized by this procedure were isolated with yields between 42–90%.

With a suite of 17 derivatives in hand, we first assessed their ability to inhibit *S. aureus* ClpP peptidase activity using an established *in vitro* assay based on the enzymatic cleavage of a fluorogenic substrate (Ac–Ala–hArg-2-Aoc–ACC, Supporting Figure S1A).[15] The compounds were tested at different concentrations (100 μM, 10 μM, 1 μM) and compared to AV170 as a positive control (Figure 3A). The most active derivatives (>50% inhibition at 1 μM) were also tested at a concentration of 100 nM (Supporting Figure S2).

Overall, five molecules, MAS-19, MAS-27, MAS-66, MAS-30, and MAS-72, were able to inhibit ClpP at concentrations below 1 μM with MAS-19 being the most active derivative with comparable potency to AV170. Closer inspection of the structure activity relationship (SAR) of all molecules indicated a clear preference of ClpP for phenyl esters containing a piperidyl scaffold equipped with an electron-deficient phenol leaving group. Remarkably, the exchange of piperidyl with cyclohexyl (MAS-55) completely abolished activity, suggesting specific interactions of the ClpP binding pocket with the piperidine nitrogen atom. The $21.5 \pm 0.6\%$ remaining activity of MAS-30 (bearing a bulky carboxybenzyl at the piperidine nitrogen) at 1 μM indicates toleration of bulky substituents in the binding pocket. Furthermore, the stronger electron-withdrawing effect of the ester substituted phenol leaving group (MAS-19) resulted in $2.20 \pm 0.3\%$ remaining activity at 1 μM, compared to almost no inhibition ($98.9 \pm 2.5\%$) in case of the lactam substituted derivative (MAS-50), indicating that electronic effects of the ester activation also play a critical role for effective acylation of the catalytic serine. In contrast, phenyl derivatives did not show effective inhibition even at 100 μM, except for MAS-21 ($21.8 \pm 0.9\%$ at 100 μM) and MAS-83 ($1.28 \pm 0.3\%$ at 100 μM). Introducing larger alkyl chains at the sulfonyl group (MAS-26) or introducing various ring substituents abrogated ClpP inhibition.
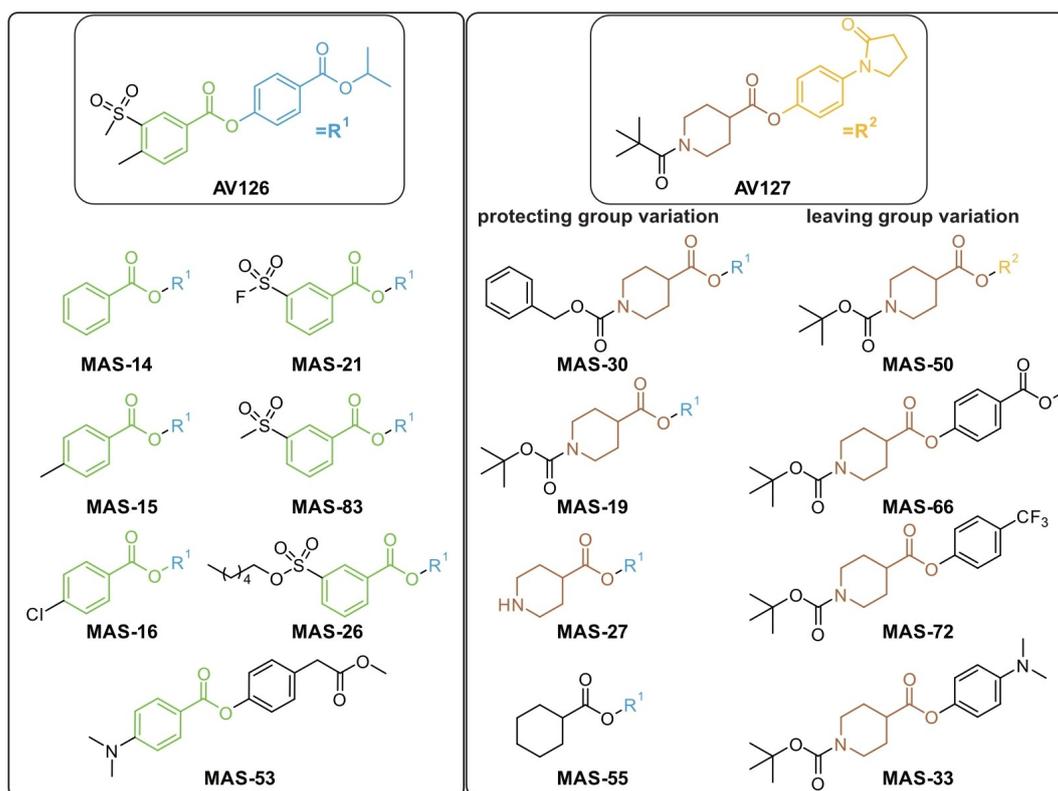
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Figure 2. Hit compounds AV126 and AV127 from the initial HTS with chemical structures of their corresponding new generation derivatives.

Binding of the client chaperone ClpX to ClpP induces conformational changes to the peptidase subunit, which enhances ClpP hydrolysis and could compromise the potency of covalent inhibitors.[13] We thus performed ClpXP protease inhibition assays with the top seven molecules and compared their performance to AV170.[14] ssrA-tagged green fluorescent-protein (eGFP) was used as substrate as its turnover relies on the formation of the whole ClpXP-complex. The molecular chaperone recognizes the C-terminal ssrA-tag, unfolds the protein, and guides it into the catalytic chamber for degradation.[14-15] All compounds were tested with the same concentrations as above and, satisfyingly, the top three compounds MAS-19, MAS-27, and MAS-30 inhibited ClpXP >50% at 1 μM, while MAS-50 and MAS-83 were largely inactive (Figure 3B). To clarify, whether inhibitory activity of MAS-50 and MAS-83 at 100 μM is based on compound inhibition or aggregation, an additional assay was performed upon addition of detergents (Supporting Figure S3). This assay proved compound inhibition to be responsible for inhibitory results. These results follow the overall trends of the ClpP peptidase assays and confirm a sufficient inhibition of the enzyme activity within the whole proteolytic complex.

With a confirmed potency against ClpXP we next tested the hydrolytic stability of our top three derivatives in phosphate-buffered saline (PBS) and determined their time-dependent hydrolysis via mass spectrometry (MS). All measurements were performed with caffeine as an internal standard based on a published procedure.[16] Incubation of 100 μM compounds in

PBS resulted in a half-life of 214 ± 14.5 min for MAS-19, which was significantly higher than AV170 (150 ± 16.3 min) followed by MAS-30 with 192 ± 18.6 min (Figure 3C). Interestingly, the half-life of MAS-27 was only 162 ± 16.2 min, suggesting that the ester group was less efficiently shielded by the unsubstituted piperidyl ring. Given the potent inhibition of ClpXP and the enhanced hydrolytic stability of MAS-19 and MAS-30, we next investigated their effect on *S. aureus* virulence compared to the AV170 benchmark. Among the virulence factors, α-hemolysin is a major *S. aureus* toxin which forms membrane-inserted homoheptamers that lyse red blood cells.[17] We aimed to investigate whether reduction of hemolysin secretion upon treatment with our novel ClpXP inhibitors is comparable to genetic *clpP* knock-out. The assay was performed on sheep-erythrocyte agar plates containing *S. aureus* NCTC 8325 co-cultured with various ClpXP inhibitors and AV170 at different doses. After overnight incubation, the production of hemolysins was quantified by erythrocyte destaining around the bacterial colony compared to the *ΔclpP* strain. Compound only and DMSO (vehicle) only controls were used to ensure that the observed hemolysis was dependent on presence of the bacteria (Figure 4A). In line with the observed trends in hydrolytic stability, MAS-19 and MAS-30 revealed the strongest reduction of hemolysis of about 50% at a dose of 250 nmol, while less stable MAS-27 resulted in a reduction of only 25%. AV170 treatment did not show any visible reduction in hemolysis (Figure 4A, 4B). Furthermore, we investigated the cytotoxicity of the top three compounds via MTT-assays in HeLa cells.
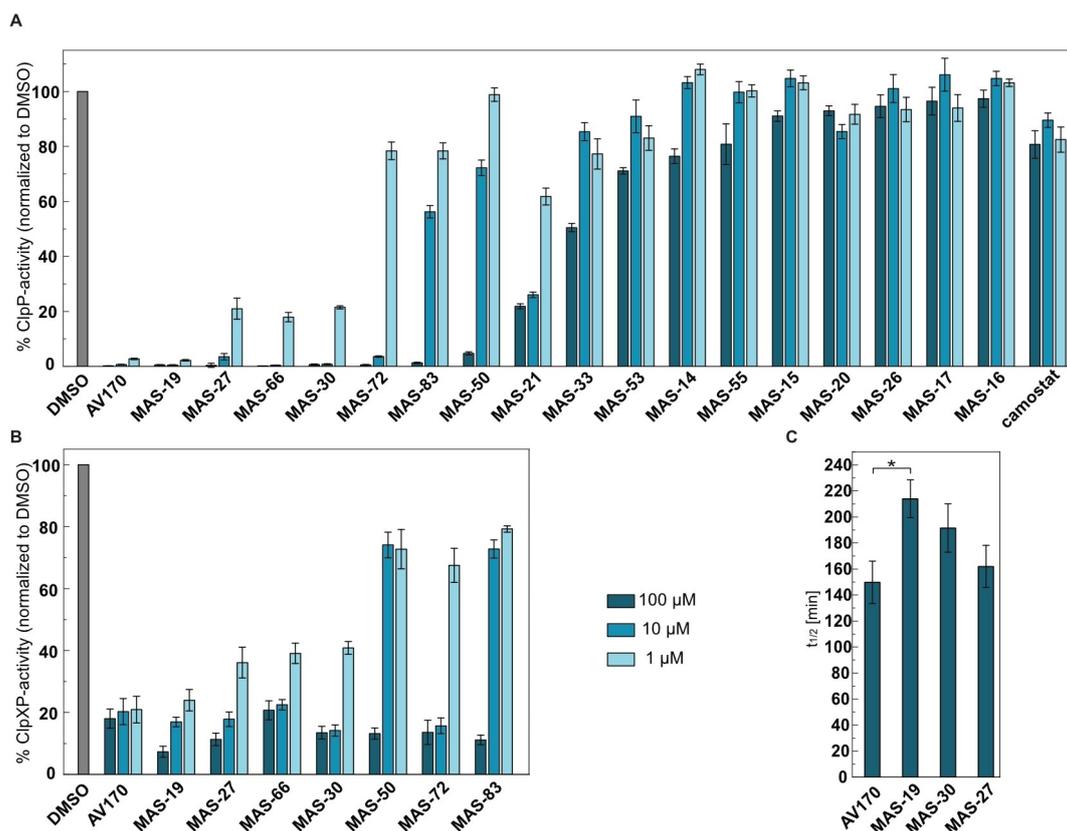
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Figure 3. (A) ClpP peptidase activity assay with fluorogenic substrate (ACC-substrate, final concentration 200 μM) at 32 °C. Remaining ClpP-activity is shown upon incubation of *Sa*ClpP (final ClpP$_{14}$-concentration: 10 nM) with 100 μM (dark blue), 10 μM (petrol) and 1 μM (light blue) of corresponding compounds compared to a DMSO control (100 % activity). The experiment was performed in at least three independent replicates ($n = 3$) in triplicates, the bars represent the mean of all 9 replicates, the error bars are SEM. (B) Investigation of degradation of ssrA-tagged eGFP (final concentration 300 nM) at 32 °C. Relative ClpXP-activity is shown upon incubation of *Sa*ClpXP (final concentrations: 100 nM ClpP$_{14}$, 200 nM ClpX$_6$) with 100 μM (dark blue), 10 μM (petrol) and 1 μM (light blue) of corresponding compounds compared to a DMSO control (100 % activity). The experiment was performed in at least two independent occasions ($n = 2$) in triplicates, the bars represent the mean of all 6 replicates, the error bars are SEM. (C) Hydrolytic stability of the top three new generation inhibitors as well as AV170 at 37 °C in PBS buffer (pH 7.4). Compounds were tested at 100 μM (initial concentration) at different time points and each data point normalized to a caffeine standard. The experiment was performed in at least three independent occasions ($n = 3$); * represents p-value ≤ 0.05 determined by Student's *t* test.

Satisfyingly, all compounds displayed minimal cytotoxicity in human cells until 100 μM. (Figure 4C). Low solubility of MAS-27 in aqueous solutions hindered accurate IC$_{50}$ determination. Finally, minimal inhibitory concentrations (MIC) could not be observed in the tested range (≤ 1 mM, not shown), highlighting potent anti-virulence activity without significant toxicity.

Conclusion

Stabilization of reactive groups to prevent premature hydrolysis is a key task in the design of covalent inhibitors. Given the potency of phenyl esters against the global virulence regulator ClpXP but their limited stability in water, we here systematically explored the phenyl ester SAR to determine if steric bulk next to the ester bond could enhance stability and thus biological activity. Based on two previously identified HTS hits, we showed that substituted piperidyl groups next to the ester paired with an electron deficient phenol leaving group not only yielded potent inhibitors of ClpP and ClpXP but also exhibited an

enhanced hydrolytic stability compared to a previously reported ClpXP inhibitor. Whether this enhanced stability can also be retained in plasma has to be investigated in future studies. These new derivatives further reduced hemolysin production, and therefore hint towards a promising anti-virulence approach. Overall, this study demonstrates not only the power of covalent ClpXP inhibitors targeting bacterial virulence but also provides guiding principles in improving their properties for biological applications.

Experimental Section

Peptidase assay: *In vitro* inhibition of *S. aureus* ClpP peptidase activity was investigated by monitoring cleavage of the optimized fluorogenic substrate peptide acetylalanyl-homoarginyl-2-amino-octanoyl-7-amino-4-carbamoylmethylcoumarin (Ac—Ala—hArg-2-Aoc—ACC, Supporting Figure S1.A, custom synthesis by *Bachem*, CH).[18] Each compound (1 μL) (100×stock solution in DMSO) or DMSO as a control was added to a black flat bottom 96-well plate (*Brand*, GER) and mixed with 98 μL of enzyme (final ClpP$_{14}$-

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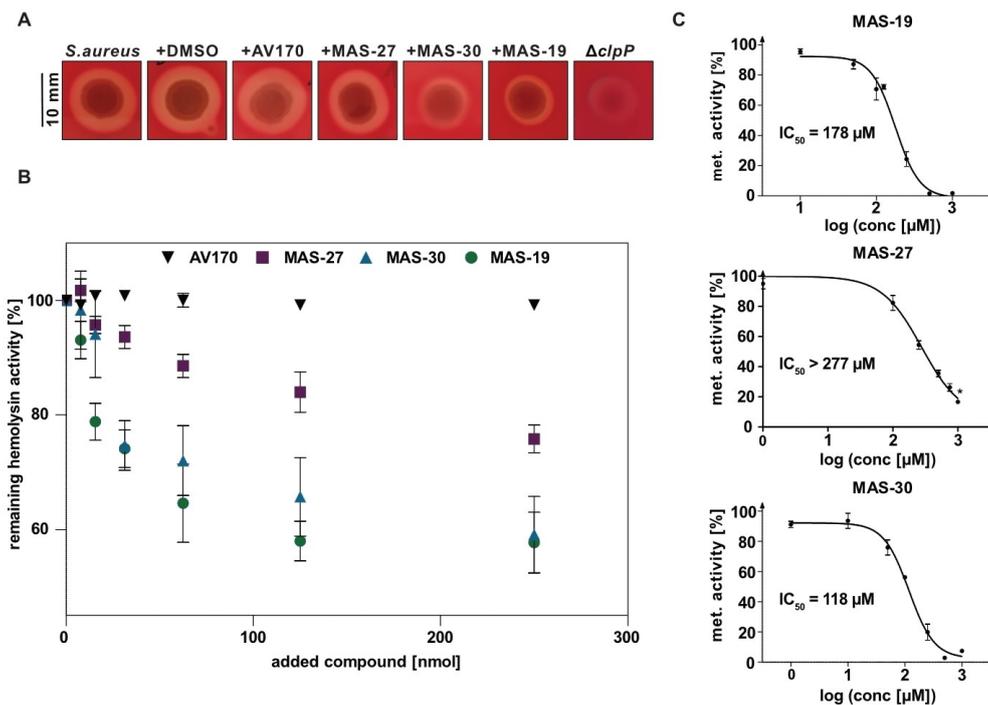Europe**
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Figure 4. (A) Representative images for de-staining of erythrocytes on sheep agar plates induced upon incubation with 250 nmol of the respective compounds or DMSO compared to untreated *S. aureus* NCTC 8325 or *ΔclpP* strain. The experiment was performed in at least 4 independent replicates (n = 4). (B) Concentration-dependent reduction of hemolysin secretion by *S. aureus* NCTC 8325 upon incubation with the respective compounds. Bacteria were co-incubated with different amounts of MAS-19 (green), MAS-30 (blue), MAS-27 (purple) and AV170 (black) overnight at 37 °C before areas of de-stained erythrocytes were determined and normalized to DMSO control. The experiment was performed in at least 4 independent occasions (n = 4). The error bars represent SEM. (C) IC$_{50}$ values for MAS-19 (top, 125–208 μM 95%CI), MAS-27 (middle, 174–439 μM 95%CI) and MAS-30 (bottom, 97–144 μM 95%CI) determined by MTT-assays in HeLa cells. The data shown corresponds to one experiment which was measured in triplicates. The experiment was performed in at least three independent occasions (n = 3) (*) Low solubility of MAS-27 in aqueous solutions hindered accurate IC$_{50}$ determination.

concentration: 10 nM) in PZ-buffer (25 mM HEPES, 200 mM KCl, 5 mM MgCl$_2$, 1 mM DTT, 10% glycerol, pH = 7.6). After incubating the mixture for 15 min at 32 °C, the reaction was started by addition of 1 μL Ac—Ala—hArg-2-Aoc—ACC (20 mM in DMSO, final concentration 200 μM). Fluorescence (λ_{ex} = 380 nm; λ_{em} = 440 nm) was monitored at 32 °C using an Infinite M200Pro plate reader (*Tecan*, AT). The initial slope of the fluorescence over time signal was calculated via linear regression. DMSO-treated samples were normalized to 100% activity. The experiment was performed in at least three independent occasions (n = 3) in triplicates.

Protease assay: *In vitro* inhibition of *S. aureus* ClpXP protease activity was investigated by monitoring cleavage of a fluorescent substrate (eGFP—ssrA), which is tagged by a short ssrA-sequence for ClpXP-mediated degradation.[15a,19] 0.6 μL of each compound (100× stock solution in DMSO) or DMSO as a control was added to a white flat bottom 96-well plate (*Brand*, GER) and mixed with 58.4 μL of enzyme mix (final concentrations: 100 nM ClpP$_{14}$, 200 nM ClpX$_6$, ATPase-regeneration system: 4 mM ATP, 16 mM creatine phosphate, 20 U/mL creatine phosphokinase in PZ buffer (25 mM HEPES, 200 mM KCl, 5 mM MgCl$_2$, 1 mM DTT, 10% glycerol, pH 7.6)). After incubating the mixture for 15 min at 32 °C, the reaction was started by addition of 1 μL eGFP—ssrA (24 μM in PZ buffer, final concentration 300 nM). GFP fluorescence (λ_{ex} = 485 nm; λ_{em} = 535 nm) was monitored at 32 °C using an Infinite M200Pro plate reader (*Tecan*, AT). The initial slope of the fluorescence over time signal in the linear range was calculated via linear regression. DMSO-treated samples were normalized to 100% activity. The experiment was performed in at least two independent occasions (n = 2) in triplicates.

Hydrolytic stability assay: Test compounds and control (caffeine) in DMSO were added at 100 μM to PBS buffer (140 mM NaCl, 10 mM Na$_2$HPO$_4$*2 H$_2$O, 2.7 mM KCl, 1.8 mM KH$_2$PO$_4$, pH 7.4) and incubated at 37 °C at 400 rpm in a thermoshaker (*Thermo Fisher*, USA). Next, 20 μL aliquots were taken after several time points, pipetted into 20 μL acetonitrile (MS-grade) and analyzed using a Dionex Ultimate 3000 HPLC system (XBridge™ BEH130 C18, 5 μm, 4.6×100 mm) coupled to a LTQ-Orbitrap XL mass spectrometer with an ESI ionization source (spray voltage: 4 kV, capillary temp.: 275 °C, capillary voltage: 24 V, tube lens: 110 V). Data was collected in positive mode using full scans (R = 60000) from m/z = 100 to m/z = 2000 and SIM scans (R = 30000) for each compound. Xcalibur 2.2 Qual Browser was used for calculation of peak areas and quantification. Results of the different time points were normalized to the control and exponential regression was used to determine half-lives of the respective compounds. The experiment was performed in at least three independent replicates (n = 3).

Hemolysis assay: Hemolysis was tested on 5% sheep blood agar plates (*Thermo Fisher*, USA) according to an established procedure.[10a] Sterile circles of Whatman® cards (No. 1, *Schleicher & Schuell*, USA) with 5.5 mm diameter were placed on the agar plates and inoculated with different amounts of respective compound stock solutions/DMSO and 2.5 μL stationary phase culture of *S. aureus* NCTC 8325 diluted to an OD$_{600}$ of 0.13 in B-medium (Peptone (10.0 g), NaCl (5.00 g), yeast extract (5.00 g), K$_2$HPO$_4$ (1.00 g) in 1 L ddH$_2$O, pH = 7.5). The plates were incubated over night at 37 °C and the diameters of the zones around the bacterial colonies were measured. Based on the corresponding diameters of the halos, the area of destained erythrocytes is calculated and

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normalized to the DMSO control and compared to the *SaΔclpP* strain. The experiment was performed in at least four independent replicates (n = 4).

Acknowledgements

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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