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# Structurally Refined β-Lactones as Potent Inhibitors of Devastating Bacterial Virulence Factors

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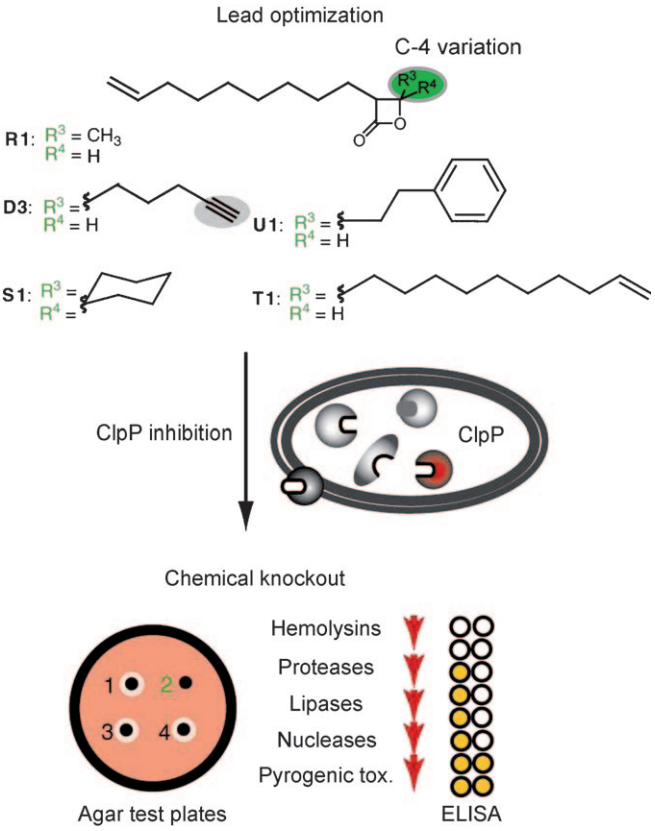
With decreasing efficiency of antibiotic therapies against hospital- and community-acquired bacterial pathogens, the treatment of infectious diseases again represents a tremendous challenge for medicinal research. This challenge seems to be particularly difficult if one considers the sophisticated resistance strategies, which are effective against almost all currently available antibiotics, developed by bacterial pathogens, such as *Staphylococcus aureus*.

The exploitation of novel antibacterial targets with low propensity for resistance, together with their corresponding inhibitors, is therefore a formidable aim for chemical and biological research.[1] Previously, we utilized a chemical proteomic strategy, called activity-based protein profiling (ABPP, pioneered by Cravatt and co-workers[2,3]), to identify functionalized β-lactones as selective and specific irreversible inhibitors of the key virulence regulator complex ClpP in *S. aureus* and corresponding methicillin-resistant (MRSA) strains.[4,5] Alternatively, ClpP can be hyperactivated by acyldepsipeptides that cause self-digestion of bacteria.[6] ClpP controls the expression of several devastating bacterial virulence factors including hemolysins, proteases and DNases, which are key players in the elimination of the host immune response and severe pain development.[7]

Most aggressive strains additionally produce pyrogenic toxin superantigens (PTSAs) such as enterotoxins and toxic shock syndrome toxin 1 (TSST-1).[8] Unlike other virulence factors, PTSAs have been found to be the direct cause of severe diseases, such as toxic shock syndrome, which is responsible for high mortality rates in infected patients. Drugs that could eliminate or reduce the expression of PTSAs would be promising candidates for the treatment of severe infectious diseases with no other current treatment options. Although no direct link has been established between PTSA expression and ClpP activity, it is assumed that at least enterotoxin B—which has even been considered as a biological warfare agent—is under its control.[9] ClpP might therefore represent such a target for inhibition of PTSA expression. Since ClpP is highly conserved in many pathogens, this strategy could represent a global approach for the treatment of infectious diseases, through disarming of the bacterial virulence repertoire and subsequent elimination by the human immune response. Targeting of virulence offers many advantages over conventional antibiotics, such as preserving the useful, cooperative microorganisms in, for example, the digestive tract, and exerting a weaker selection pressure on pathogens, which could result in decreased resistance.[10,11]

In previous experiments we were able to show that one of our β-lactone probes (**D3**, Scheme 1) was able to abolish the hemolytic and proteolytic activities of *S. aureus* and significantly reduced their expression in MRSA through ClpP inhibition; this confirmed previous results obtained with a ClpP knockout mutant.[5,7] Because the efficiency of **D3** greatly exceeded that of other β-lactones in our initial library, we speculated that structural refinement of this lead compound might yield even higher potency for ClpP inhibition. Here, we present an optimized inhibitor for ClpP and investigate its ability to abolish the production of the devastating PTSAs and other crucial virulence factors.




**Scheme 1. D3** is a lead compound for ClpP labeling and inhibition. Synthetically refined compounds of **D3** were tested for improved ClpP inhibition and for their biological effects on virulence factor expression.

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We started with the chemical refinement of the long-chained aliphatic β-lactone **D3** through the synthesis of novel derivatives, by substitution of the alkyne linker in the C4 position with structurally diverse moieties (Scheme 1). In our previous library the alkyne linker was an invariant residue that was used for the introduction of a rhodamine or biotin tag after in vivo target binding (by ABPP click chemistry for SDS-PAGE and avidin bead affinity enrichment[4]).

To investigate whether the alkyne moiety is an essential component for ClpP inhibition, the substitution at the C4 position was varied with a selection of ligands including small (**R1**), cyclic (**S1**), aliphatic (**T1**) and aromatic (**U1**) residues (Scheme 1). To estimate the individual potencies of the new β-lactone inhibitors for ClpP-binding, intact *S. aureus* cells were incubated under in vivo conditions at various concentrations of **R1**, **S1**, **T1** and **U1**. After cell lysis the **D3** probe was added in order to label the remaining unbound active sites. Subsequent click chemistry[12–14] with rhodamine azide and fluorescent gel analysis allowed the determination of the strength of the inhibitor–enzyme interaction—potent inhibitors should lead to significant decreases in the **D3** fluorescence signal. In fact, **R1** and **U1** were able to reduce **D3** labeling significantly (Figure S1 in the Supporting Information). This is consistent with peptidase activity assays of recombinant ClpP, which was inhibited by **R1** with an $EC_{50}$ of 3 μM and by **U1** with an $EC_{50}$ of 7 μM (Figure S2 in the Supporting Information). However, the hydrophobic nature of **U1** caused solubility problems in the assay buffer and restricted the evaluation of full peptidase inhibition (although detection of $EC_{50}$ for **U1** was possible). No inhibition of ClpP activity could be observed for **S1** and **T1** below 40–60 μM (these compounds showed poor solubilities at higher concentrations).

The biological potencies of all new compounds and their effects on virulence factor expression were tested by several agar-plate-based experiments with which pathogen-mediated hemolysis and proteolysis as well as lipase and DNase activities could be easily visualized. Hemolysins are critical virulence factors of *S. aureus* and disrupt sheep erythrocytes mainly through the action of α- and β-toxins.[15] These toxins are the major causes of brain abscess development and scleral inflammation, respectively.[16,17] Hemolysis assays with antibiotic sensitive *S. aureus* NCTC 8325 revealed that inhibitor **U1**, which has a phenylethyl group in position C4, exhibited greatly increased potency (4.9-fold) with an effective dose ($ED_{50}$) of 7 nmol relative to the best previous lactone **D3** ($ED_{50}$ = 34 nmol); all other inhibitors were biologically less active (Figures 1A and B, Figures S3 and S4 in the Supporting Information).

Although these results are encouraging, the major challenge in antibacterial research is the treatment of devastating infec-

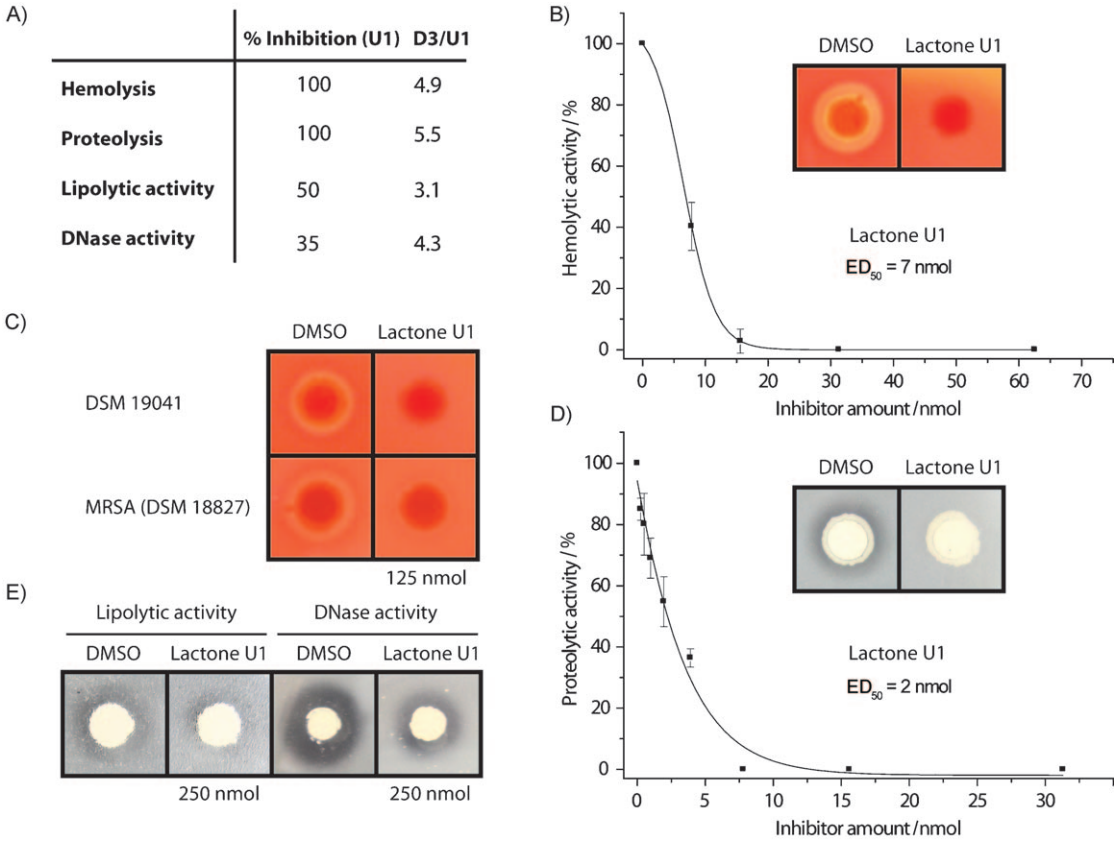


**Figure 1.** Inhibition of extracellular virulence by β-lactones. A) Inhibition of virulence factors and the relative improvement of the structurally refined inhibitor **U1** (250 nmol) relative to the most potent β-lactone (**D3**) of the initial ABPP probe generation. B) Inhibition of hemolysis in antibiotic-sensitive *S. aureus* NCTC 8325, and C) inhibition of hemolysis in the MRSA strain DSM 18827 and the toxin-producing strain DSM 19041. D) Inhibition of proteolysis in NCTC 8325 by lactone **U1** as determined by agar-plate-based assays, and E) partial inhibition of lipolytic and DNase activities.

  

tions caused by enterotoxin-producing and multiply resistant *S. aureus* strains. We therefore investigated the effects of ClpP inhibition by **U1** in a methicillin-resistant *S. aureus* strain (MRSA, DSM 18827) and in a highly virulent toxin-producing clinical *S. aureus* isolate (DSM 19041). Indeed, treatment of both strains with **U1** (125 nmol) resulted in complete inhibition (clinical isolate) as well as significantly reduced (80%) hemolysis (MRSA; Figures 1C and S5 in the Supporting Information).

Compound **T1**, which is the most bulky lactone of the optimized generation, was the only one that had virtually no effect on hemolysis (Figure S4 in the Supporting Information); this indicates that aromatic residues at the C4 position are preferred ligands for ClpP binding.

Because our synthetic route provided the *trans*-β-lactone **U1** as a racemic mixture, we were interested to see whether it was one or both enantiomers that contributed to ClpP inhibition. We therefore separated the two enantiomers by chiral HPLC (see the Supporting Information) and tested them separately in hemolysis assays. Enantiomer 1 showed slightly reduced (1.3-fold) antihemolytic potency in relation to enantiomer 2, the activity of which was comparable to that of the racemate (Figure 2). The racemic mixture was therefore at least equal to the better single enantiomer in potency.

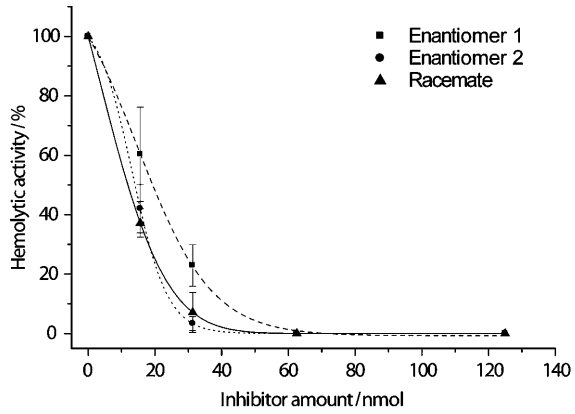


**Figure 2.** Agar plate hemolysis assays for both enantiomers and for the racemic mixture of lactone **U1** with *S. aureus* NCTC 8325.

Proteolysis is an additional crucial virulence strategy used by many pathogens for tissue invasion and escape from the host immune response.[18] Corresponding assays again revealed **U1** to be the most potent compound ($ED_{50} = 2$ nmol, 5.5-times more potent than **D3**) for the full inhibition of extracellular proteolysis (Figure 1A and D). In addition, DNases and lipases are important for bacterial evasion of host response. Staphylococcal lipase, for instance, has been demonstrated to strongly reduce the phagocytic killing of *S. aureus* by granulocytes.[19] Application of **U1** to *S. aureus* grown on test agar showed significantly reduced lipolytic and DNase activities (Figures 1A and E, Figure S6 in the Supporting Information). However, basal levels of both exoenzyme activities were observed even at the highest lactone doses. Compounds **T1** and **M1**, which is a control lactone that does not inhibit ClpP, again showed no effect in these tests (Figure S4 in the Supporting Information).

Finally, we investigated the effect of **U1** on *S. aureus* PTSA expression, which is the direct cause of severe diseases, such as staphylococcal food poisoning (SFP) and toxic shock syndrome (TSS).[8] Unlike other virulence factors, the regulation of these toxins has not been addressed in previous genetic ClpP knockout experiments. Here, we show through a sandwich ELISA immunoassay toxin test (see the Supporting Information) that chemical ClpP knockout by treatment with **U1** (2.5 mM) resulted in significantly reduced secreted protein levels of important toxins, including enterotoxin B (SEB, 68% reduction) in strain DSM 19041 and enterotoxin C3 (SEC3, 89% reduction) in the MRSA strain Mu50 (Figure 3). Unlike enterotoxin A (SEA),

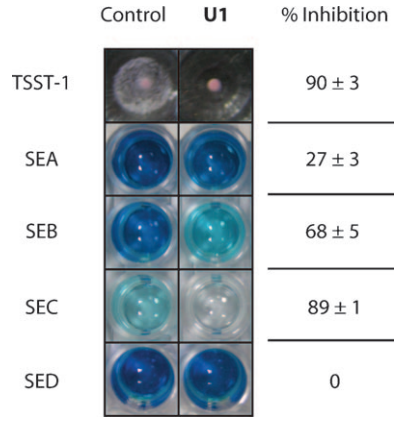


**Figure 3.** Immunoassays of inhibition of important *S. aureus* pyrogenic toxins. Immunoassays with filtered bacterial culture supernatants in the presence (control) and absence of lactone **U1** (2.5 mM). Inhibition (%) was determined for enterotoxins A (SEA), B (SEB), C (SEC) and D (SED) and for toxic shock syndrome toxin 1 (TSST-1). Strong effects were found for SEB (strain DSM 19041) and for SEC and TSST-1 (MRSA strain Mu50).

which was not significantly inhibited, SEB and SEC3 belong to the same group of enterotoxins and constitute—together with toxic shock syndrome toxin 1—the most relevant PTSAs for infectious diseases. Interestingly, a significant reduction in TSST-1 expression of 90% after treatment with 2.5 mM **U1**, and even 75% with only 156 μM **U1**, was observed in the MRSA strain Mu50 as shown by a reverse passive latex agglutination TST-RPLA test (Figure S7 in the Supporting Information). These results emphasize the unprecedented utility of the customized ClpP inhibitor **U1** for global inhibition of virulence in *S. aureus*. This is especially important with regard to the untreatable MRSA and virulent strains such as Mu50 and DSM 19041, which produce high levels of PTSAs.

In conclusion, we have optimized the structure of a previously identified long-chained aliphatic β-lactone probe for *S. aureus* ClpP binding by screening diverse ligands at the lactone C4 position. Inhibitor **U1**, with a phenylethyl side chain in the C4 position, represented the best candidate out of this focused screen and showed four- to fivefold increased potency in the reduction of crucial virulence factor expression levels, including the devastating PTSAs. The decoration of this inhibitor

  

therefore seems to resemble the active site requirements of ClpP and it might be a promising candidate for further investigation of its role in virulence regulation. The phenotypic properties after lactone treatment exactly correspond to the observed down-regulation of virulence factors reported for a *ΔClpP* mutant. So far, however, PTSA-producing strains have not been subjected to genetic ClpP knockout studies. Our results indicate that SEB, SEC3 and TSST-1 are also under the control of ClpP. Future in vivo studies should reveal the potential use of ClpP inhibitors in antibacterial therapy. In fact, targeting of this virulence regulator might represent an attractive strategy for neutralizing the harmful effects of bacterial pathogens and help the host immune response to eliminate the disarmed bacteria. Previous studies have already shown that a *S. aureus* ClpP knockout strain displays significantly reduced pathogenesis in a murine skin abscess model.[7]

## Experimental Section

**Synthesis of β-lactones**: β-Lactones were prepared from *S*-phenyl-undec-10-enethioate and various aldehydes as described in the Supporting Information.

**Competitive ClpP labeling**: For competitive ClpP labeling experiments, live cells were incubated at various lactone concentrations (or with DMSO for control experiments) for 2 h at room temperature and were washed three times with PBS (5 mL) to remove excess lactone. Cells were lyzed by sonication in PBS (1 mL) and the cytosolic fractions were adjusted to protein concentrations of 1 mg mL$^{-1}$. Compound **D3** (50 μM) was added to each sample and the mixtures were incubated for 1 h at room temperature, followed by addition of click chemistry (CC) reagents.

**$EC_{50}$ measurement**: ClpP $EC_{50}$ values for the different lactones were measured through inhibition of the turnover of the fluorogenic substrate *N*-succinyl-Leu-Tyr-7-amidomethylcoumarin (Succ-Leu-Tyr-AMC).

**Biological assays**: Hemolysis was tested with sheep blood (5%) agar plates and proteolysis was assessed on lysogenic broth (LB) agar plates containing skimmed milk (1%). Lipolytic activity was tested on tributyrin agar (Sigma–Aldrich) with emulsified tributyrin (1%) and DNase activity was assessed on DNase test agar. Sterile circles of Whatman® cards (5.5 mm diameter) were placed on the agar plates and inoculated with the corresponding lactone/DMSO (2.5 μL) and a dilution of a stationary phase culture of *Staphylococcus aureus* (2.5 μL). Plates were incubated, overnight, at 37 °C, and the zones appearing around the circles were quantified. For PTSA testing, immunoassays were performed with sterile filtered culture supernatants of *S. aureus* liquid cultures grown with lactone **U1** or DMSO to stationary phase. SEA, SEB, SEC3 and SED were examined by using a sandwich ELISA immunoassay and TSST-1 by reverse passive latex agglutination.

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