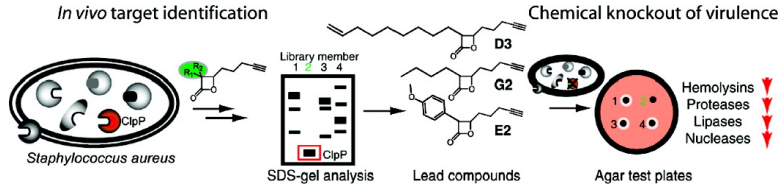
Communication

#-Lactones as Specific Inhibitors of ClpP Attenuate the Production of Extracellular Virulence Factors of *Staphylococcus aureus*

Thomas Bo#ttcher, and Stephan A. Sieber

J. Am. Chem. Soc., **2008**, 130 (44), 14400-14401 • DOI: 10.1021/ja8051365 • Publication Date (Web): 11 October 2008



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

J|A|C|S
COMMUNICATIONS

Published on Web 10/11/2008

β-Lactones as Specific Inhibitors of ClpP Attenuate the Production of Extracellular Virulence Factors of *Staphylococcus aureus*

Thomas Böttcher and Stephan A. Sieber*

Center for integrated Protein Science Munich CIPS[M], Department of Chemistry and Biochemistry, Ludwig-Maximilians-Universität München, Butenandtstrasse 5-13, 81377 Munich, Germany

Received July 15, 2008; E-mail: stephan.sieber@cup.uni-muenchen.de

With the evolution of multidrug resistant bacterial pathogens, infectious diseases pose once again a serious threat to public health. Especially the opportunistic pathogen *Staphylococcus aureus* has gained importance through the dramatically increasing appearance of methicillin-resistant (MRSA) strains in hospitals and the recent emergence of epidemic community-associated MRSA infections.[1] Major reasons for this daunting problem are the excessive use of conventional antibiotics, the limited number of essential cellular targets addressed by these compounds, and their paramount selective pressure exerted on bacterial viability leading to resistance development. Here we introduce an alternative strategy based on functionalized β-lactones to target bacterial virulence rather than viability. A central role in *S. aureus* virulence regulation can be attributed to the caseinolytic protein protease (ClpP), a phylogenetically highly conserved serine protease that was found to be crucial for virulence of many bacterial pathogens.[2]

The role and importance of ClpP for virulence has been demonstrated by a ΔClpP mutation in *S. aureus* which exhibited strongly decreased extracellular virulence.[2] The molecular reasons for the phenotypic properties of a ΔClpP mutant are attributed to the up-regulation of several transcriptional repressors of virulence genes (SarA family) and concomitant down regulation of the virulence inducing *agr* quorum sensing system (Supporting Information, Figure S1).[3] Selective inhibition of ClpP by a new generation of drugs may thus represent a novel strategy in the treatment of infectious diseases. Previously, ClpP has been targeted by acyldepsipeptides which led to the hyperactivation and proposed self-digestion of bacteria.[4] However, no effective inhibitor of ClpP has been reported up to now, including our initial attempts with β-lactams.[3,5] Here we show that selective inhibition of this central virulence regulator in *S. aureus* and MRSA by synthetic *trans*-β-lactones resulted in a drastically decreased expression of major virulence factors including hemolysins, proteases, DNases, and lipases, which are key players in the elimination of the host immune response, tissue necrosis, and inflammation.

Recently, we demonstrated that a selection of *trans*-β-lactone probes which were screened in activity-based protein profiling (ABPP, pioneered by Cravatt) experiments[6,7] are cell permeable and can specifically label ClpP of nonpathogenic bacterial strains in situ.[8] Inspired by these results, we here apply these molecules to *S. aureus* and MRSA strains aiming to (1) investigate the labeling of ClpP and potential off-targets in living cells of a pathogenic organism, (2) quantitatively assess the potency of individual compounds by their ability to inhibit ClpP, and (3) search for effects on extracellular virulence factors by chemical knockout experiments with living cells.

First, the small biomimetic library of seven β-lactone probes was applied to living *S. aureus* cells under in situ conditions (Figure 1B). After cell lysis, an azide-rhodamine tag was appended to the labeled proteins of the cytosolic proteome fraction via click chemistry (CC) prior to SDS PAGE analysis. Gel bands of labeled proteins were detected by fluorescence scanning and identified by mass spectrometry. Interestingly, three probes, **D3** and **G2** with aliphatic moieties and **E2** with a small aromatic moiety were capable of labeling *S. aureus* ClpP, which is a low abundance cytosolic enzyme, in great intensity and as the main target (Figure 2A). At concentrations below 100 μM, the probes exerted a remarkable selectivity for ClpP which was the exclusive target inside the cell with no additional binding partners in membrane and extracellular proteomes. Subsequent in situ dose-down experiments revealed that the sensitivity increased in the order **G2** < **E2** ≪ **D3** and concentrations as low as 1.3 μM of **D3** were sufficient for ClpP labeling and detection (Figure 2B). Saturation of ClpP labeling was

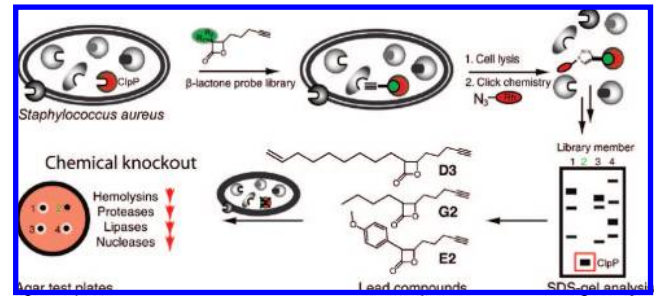


Figure 1. A chemical proteomic strategy for the in situ labeling of ClpP by cell-permeable β-lactone probes and subsequent identification by fluorescent SDS-gel analysis and mass-spectrometry. Lead compounds for ClpP labeling and inhibition are tested for their biological effects on virulence factor expression.

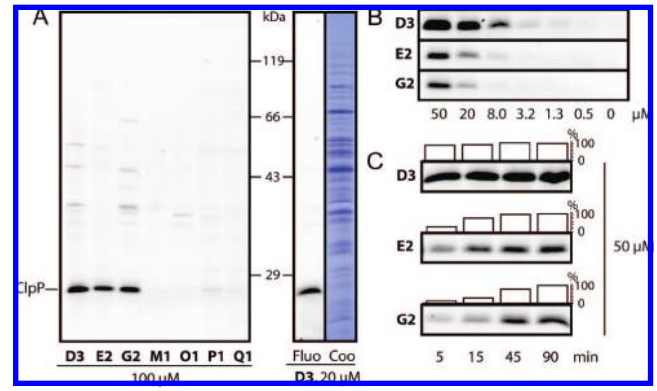


Figure 2. Identification of potent β-lactone probes for ClpP labeling in *S. aureus*. (A) Only three probes **D3**, **E2**, and **G2** show specific labeling of ClpP. Comparison of fluorescence (Fluo) scanning and Coomassie (Coo) staining at 20 μM probe concentration reveals extraordinarily high specificity of **D3** for the low abundance protein ClpP. (B) In situ dose-down experiments show the highest sensitivity of ClpP labeling for **D3** followed by **E2** and **G2**. (C) Varying incubation times indicate that target saturation is reached almost immediately for **D3** in contrast to **E2** and **G2**.

10.1021/ja8051365 CCC: $40.75

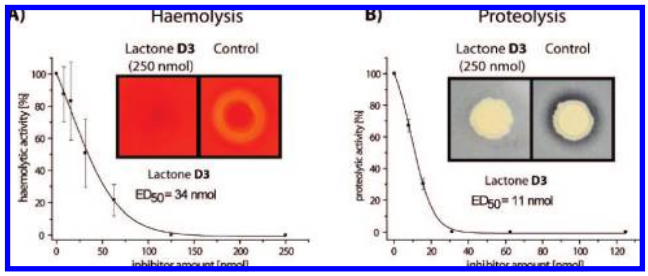


Figure 3. Inhibition of extracellular virulence by β-lactones: (A) inhibition of hemolysis and (B) of proteolysis by **D3** with agar plate based assays.

almost reached immediately (90% in 15 min) for **D3**, whereas 45 min were required for **E2** and ≥90 min for **G2**, emphasizing that the long chained hydrophobic lactone **D3** is suited best to penetrate the cellular lipid bilayer (Figure 2C).

To test whether labeling of the native ClpP complex is active-site directed and inhibits the peptidase activity, *S. aureus* ClpP and an active site Ser98Ala mutant were recombinantly expressed. Native ClpP was purified and used in a fluorescence peptidase activity assay.[9] All three β-lactones were able to impair the peptidase activity of ClpP and completely inhibited substrate cleavage. While **G2** exerted the weakest effect on peptidase activity with an IC_{50} of 31 μM, the β-lactones **D3** and **E2** showed a much stronger inhibitory effect and were comparable regarding their IC_{50} values of 6 and 4 μM, respectively (Figure S2). Probe **M1** for which no labeling of ClpP was observed did not reveal any inhibition of peptidase activity even at high concentrations of 1.6 mM. The mutant enzyme was not labeled by **D3** confirming Ser98 as the catalytic nucleophile for D3 binding (Figure S3).

The successful inhibition and in situ labeling of ClpP by β-lactones raised the question whether these molecules could also impair its natural function, leading to a reduced production of virulence factors as reported for a ΔClpP mutant *S. aureus* strain.[2] Hemolysins are critical virulence factors of *S. aureus*, disrupting erythrocytes mainly by the action α- and β-toxins.[10] These toxins are the major cause of brain abscess development and scleral inflammation, respectively.[11,12] Indeed, agar plate-based assays with 5% sheep blood showed inhibition of hemolysis in *S. aureus* NCTC 8325 for lactones **D3**, **E2**, and **G2** while the control-probe **M1** had no effect (Figure 3,and Figures S4, and S5). The relative efficiency of the probes corresponded well with the in situ labeling intensities with **G2** < **E2** ≪ **D3**. Compound **D3** was able to even completely abolish hemolysis at doses above 125 nmol.

Proteolysis represents an additional crucial virulence strategy used by many pathogens for tissue invasion and escape from the host immune response.[13] Corresponding assays on 1% skim milk agar plates revealed again **D3** as the most potent compound (ED_{50} = 11 nmol,) for the full inhibition of extracellular proteolysis (Figures 3 and S4).

In addition, DNases and lipases are important for bacterial evasion of host response. Staphylococcal lipase for instance was demonstrated to highly reduce the phagocytic killing of *S. aureus* by granulocytes.[14] Application of **D3** to *S. aureus* grown on test agar showed significantly reduced lipolytic and DNase activities (Figure S6). However, a basal level of both exoenzyme activities was observed even at the highest lactone doses, which suggests that a subset of lipases and DNases are either less tightly regulated by ClpP or not under its control. **M1** again showed no effect in these tests (Figure S5). Interestingly, the phenotypic properties of our chemical ClpP knockout in *S. aureus* exactly match those reported for the genetic ΔClpP mutant (Table S1).[2] However, the challenge of current antibacterial research is the treatment of infections caused by highly pathogenic MRSA strains for which no ClpP knockout has been reported up to now. To investigate whether ClpP inhibition represents a global strategy for virulence factor attenuation, we tested the effect of **D3** on hemolytic and proteolytic activities in MRSA clinical isolates (DSM 18827 and Mu50, respectively). Indeed, hemolytic and proteolytic activities were significantly reduced by 70% and 100%, respectively, which suggests a central role of ClpP even in resistant strains and emphasizes the value of β-lactones as putative lead structures for MRSA treatment (Figure S7).

In conclusion, we have identified synthetic β-lactones as novel inhibitors for specific and selective targeting of the key virulence regulator ClpP in *S. aureus* and in MRSA strains. In fact, our most potent inhibitor **D3** was able to completely abolish hemolytic and proteolytic activities and showed a dramatic decrease in the expression of lipase and DNase activities. Targeting this virulence regulator may therefore represent an attractive strategy for neutralizing the harmful effects of bacterial pathogens and help the host immune response to eliminate the disarmed bacteria. Indeed, previous studies have already shown that a *S. aureus* ClpP knockout strain displayed significantly reduced pathogenesis in a murine skin abscess model.[2] Since ClpP is not essential for viability and highly conserved in many pathogens, our strategy could represent a global approach for the treatment of infectious diseases minimizing the selective pressure on bacteria and resistance development.[15]

Acknowledgment. We thank Prof. Thomas Carell for his generous support and acknowledge funding by the Emmy Noether program and SFB 749 of the DFG, Römer-Stiftung, Fonds der chemischen Industrie, CiPS[M], and Studienstiftung des deutschen Volkes (T.B.). We thank Dr. Michael Schloter (GSF) for the use of his S2 laboratory and Kerstin Kurz for excellent technical assistance.

Supporting Information Available: Proteome preparation and labeling, virulence assays, and enzyme inhibition. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Burlak, C.; Hammer, C. H.; Robinson, M. A.; Whitney, A. R.; McGavin, M. J.; Kreiswirth, B. N.; Deleo, F. R. *Cell Microbiol.* **2007**, *9*, 1172–90.
(2) Frees, D.; Qazi, S. N.; Hill, P. J.; Ingmer, H. *Mol. Microbiol.* **2003**, *48*, 1565–78.
(3) Michel, A.; Agerer, F.; Hauck, C. R.; Herrmann, M.; Ullrich, J.; Hacker, J.; Ohlsen, K. *J. Bacteriol.* **2006**, *188*, 5783–96.
(4) Brötz-Oesterhelt, H.; Beyer, D.; Kroll, H. P.; Endermann, R.; Ladel, C.; Schroeder, W.; Hinzen, B.; Raddatz, S.; Paulsen, H.; Henninger, K.; Bandow, J. E.; Sahl, H. G.; Labischinski, H. *Nat. Med.* **2005**, *11*, 1082–7.
(5) Staub, I.; Sieber, S. A. *J. Am. Chem. Soc*. 2008, in press.
(6) Evans, M. J.; Cravatt, B. F. *Chem. Rev.* **2006**, *106*, 3279–301.
(7) Speers, A. E.; Adam, G. C.; Cravatt, B. F. *J. Am. Chem. Soc.* **2003**, *125*, 4686–4687.
(8) Böttcher, T.; Sieber, S. A. *Angew. Chem., Int. Ed.* **2008**, *47*, 4600–3.
(9) Maurizi, M. R.; Thompson, M. W.; Singh, S. K.; Kim, S. H. *Methods Enzymol.* **1994**, *244*, 314–31.
(10) Wiseman, G. M. *Bacteriol. Rev.* **1975**, *39*, 317–44.
(11) Walev, I.; Weller, U.; Strauch, S.; Foster, T.; Bhakdi, S. *Infect. Immun.* **1996**, *64*, 2974–9.
(12) O'Callaghan, R. J.; Callegan, M. C.; Moreau, J. M.; Green, L. C.; Foster, T. J.; Hartford, O. M.; Engel, L. S.; Hill, J. M. *Infect. Immun.* **1997**, *65*, 1571–8.
(13) Lowy, F. D. *New Engl. J. Med.* **1998**, *339*, 520–32.
(14) Rollof, J.; Braconier, J. H.; Soderstrom, C.; Nilsson-Ehle, P. *Eur. J. Clin. Microbiol. Infect. Dis.* **1988**, *7*, 505–10.
(15) Clatworthy, A. E.; Pierson, E.; Hung, D. T. *Nat. Chem. Biol.* **2007**, *3*, 541–548.

JA8051365