Enzyme Inhibitors

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The Mechanism of Caseinolytic Protease (ClpP) Inhibition**

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Maintaining homeostasis at the protein level is an important prerequisite for cellular viability for which prokaryotes exhibit several proteolytic machineries, including ClpXP.[1] In 2008, we reported the first small-molecule inhibitor for the proteolytic subunit ClpP and demonstrated that the inhibition of the enzyme in living bacteria significantly attenuates their capability to produce virulence factors, such as life-threatening toxins.[2] Although ClpP has been extensively studied by biochemical and structural methods,[3] the mechanism of small-molecule inhibition of this enzyme is currently poorly understood. Because chemical inhibition may lead to a novel antibacterial therapy, it is important to systematically analyze the binding site, the mechanism of inhibition, the stereogenic preference of the enzyme for inhibitors, the chemical space of putative inhibitors, and how other members of the ClpP family can be inhibited. One major step towards these aims was accomplished by the recently solved crystal structure of homotetradecameric ClpP from *Staphylococcus aureus* (SaClpP) in its active conformation.[4] With the structural data at hand, we herein report an in-depth mechanistic analysis of *S. aureus* ClpP inhibition by β-lactones. A screen of a focused library of enantiopure β-lactones revealed the *S*,*S*-stereopreference of the protease, which was rationalized by molecular docking. Docking experiments also gave insight into a hitherto unnoted deep hydrophobic pocket next to the active site that accommodates β-lactone substituents in the α-position to the carbonyl group. The binding hypothesis was verified by binding studies with model compounds, detailed kinetic analysis, and protein mutagenesis studies. Furthermore, the replacement of the β-lactone core by other scaffolds resulted in the loss of inhibitory potency, thereby highlighting the importance of a β-lactone moiety for mechanism-based ClpP inhibition. Taken together, these results open intriguing perspectives in the mechanistic understanding of ClpP inhibition and provide direction for the design of potent and pharmacologically optimized inhibitors.

We started by testing 22 enantiopure *trans*-substituted β-lactones **1**–**22** for ClpP inhibition (Supporting Information, Figure S1 A).[5] These molecules share a high structural similarity with our previous β-lactone candidates. They feature a decyl chain as R^1 substituent and structural variations in chain lengths as well as in functional groups at the R^2 position (Figure 1 A). For all of the compounds, both *trans*-configured enantiomers (that is, *R*,*R* and *S*,*S*) were tested for inhibition of recombinantly expressed SaClpP in an assay monitoring the cleavage of a fluorogenic substrate.[4] Almost all of the compounds inhibited SaClpP at 100 μM concentration (100-fold excess over enzyme) after 15 min incubation at 32 °C (Supporting Information, Figure S1 A). By lowering the inhibitor concentration to 10 μM, we were able to differentiate the compounds tested. While most *S*,*S*-configured lactones lead to inhibition below 10 % residual activity, *R*,*R*-configured lactones showed essentially no inhibition (Figure 1 B). Incubation of SaClpP with 1.3-fold molar excess of the most potent compound, **2**, led to modification of all 14 subunits as revealed by intact-protein mass spectrometry (Figure 1 C).

To investigate if the potent in vitro inhibition correlates with ClpP binding in living cells we applied the structurally related alkynylated probe **23** with *S*,*S*-configuration for an

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Supporting information for this article (detailed procedures for chemical syntheses, biochemical methods, and docking experiments) is available on the WWW under http://dx.doi.org/10.1002/anie.201204690.

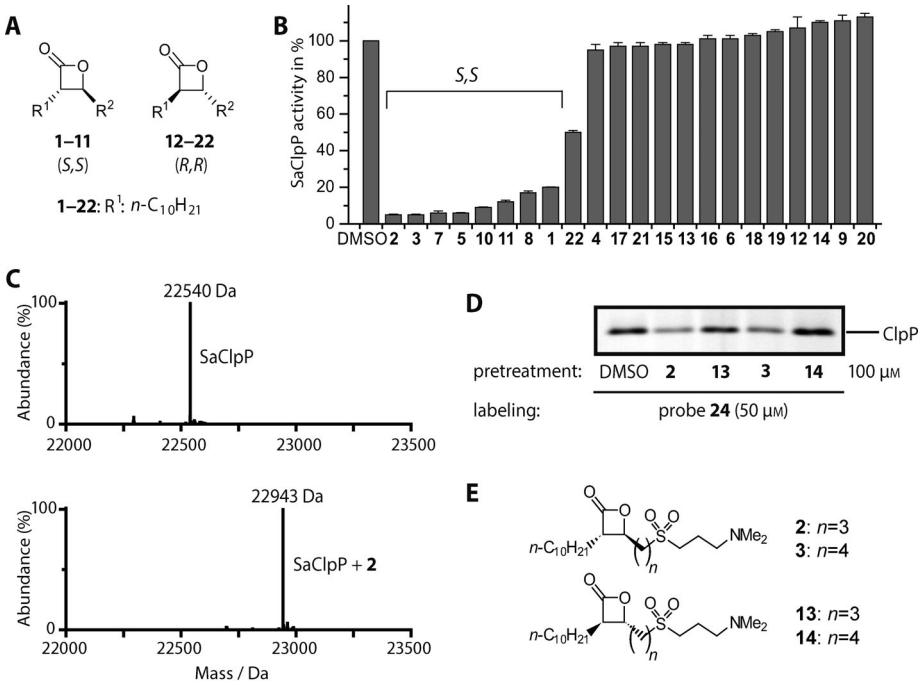


Figure 1. A) General formula of the investigated β-lactones with *S,S* or *R,R* stereochemistry (**1–11** and **12–22**, respectively). R^1 is *n*-$C_{10}H_{21}$; R^2 is varied. (See the Supporting Information, Figure S1 for a complete list of structures.) B) Residual activity of SaClpP (1 μM) after incubation with compounds **1–22** (10 μM) for 15 min at 32 °C. C) Intact-protein mass spectrometry, which reveals inhibition of all 14 SaClpP subunits by compound **2** (403.3 Da, 1.3 molar excess). D) Competitive labeling experiment. *S. aureus* NCTC 8325 cells were preincubated with indicated compounds at 100 μM for 1 h at room temperature. Labeling was performed with 50 μM of ClpP-specific probe **24** for 1 h. Following lysis, rhodamine azide was appended to **24** by bioorthogonal click chemistry and the proteome was separated by SDS-PAGE. See the Supporting Information, Figure S2 for a full image of the gel, and the Supporting Information, Figure S4A for loading controls. E) Structures of compounds **2** and **3** as well as **13** and **14** used in the competitive labeling experiment.

in situ labeling of *S. aureus* NCTC 8325 (Supporting Information, Figure 2A).[5b,6] After incubation and cell lysis, a fluorescent tag was attached by click chemistry[7] and the proteome was analyzed by SDS-polyacrylamide gel electrophoresis. A fluorescent band was observed at the same height as for the ClpP-specific probe **24**, indicating that the physiological target of the inhibitors tested is ClpP. A much weaker band was observed for the *cis*-configured analogue of probe **24**,[2a] which is indicative of a strong preference for *trans*-configured β-lactones (Supporting Information, Figure S2B). Pretreatment of living *S. aureus* cells with *S,S*-configured lactones **2** and **3** followed by labeling with probe **24** lead to a decrease in fluorescence intensity of the ClpP band, while the respective *R,R*-configured enantiomers **13** and **14** showed no effect (Figure 1D,E). This competitive labeling experiment confirms the physiological relevance of the in vitro data. Next, we utilized molecular docking studies to unravel the binding mode and rationalize the observed preferences for long chain aliphatic substituents and *S,S*-configured lactones.

β-Lactones[8] are known to react covalently with the catalytic Ser98,[2b] which leads to ring opening and blockage of the active site and furnishes a catalytically inactive β-hydroxyacyl–enzyme complex. To determine the geometry of the acyl–enzyme complex, a covalent docking was performed with all known inhibitors into the crystal structure of active SaClpP (Figure 2A,B).[4] Remarkably, all well-ranked docking solutions shared the placement of the hydrophobic R^1 chain in a deep pocket of the protein, adjacent to the active site (Figure 2B; Supporting Information, Figure S3A). While the opening of the pocket is narrow and corresponds to the S1 pocket of the protease,[9] the channel widens into an enclosed cavity, the physiological role of which is unclear. The occupation of this channel along with the enclosed cavity by a ligand most likely results in the replacement of water molecules and reduces the solvent accessible surface by about 165 Å^2, of which 65 % is apolar (Supporting Information, Table S1, Figure S3B).[10] The binding of the hydrophobic R^1 ligand portion in the pocket possibly provides the driving force for the initial protein–inhibitor association, prior to a fast nucleophilic attack of Ser98 that is due to spatial proximity to the β-lactone core.[11]

To map the space available to accommodate ligand side chains, we performed covalent docking using a virtual β-lactone library consisting of compounds with aliphatic chains as R^1 substituents covering a chain length from one to twelve carbon atoms (Figure 2C). Scoring values increased with the length of the substituent with a maximum at eight atoms. Only side chains with up to ten atoms could be accommodated in the pocket (Figure 2C). Next, we sought to validate these results experimentally by an inhibition assay. IC_{50} values of covalent inhibitors highly depend on the incubation time and the enzyme concentration used.[12] We therefore used $k_{obs}/[I]$ values as a quantitative measure of potency.[13] We synthesized compounds **25**, **27–29** and developed an additional fluorogenic substrate assay in which the increase in fluorescence during inhibition was directly monitored and from which rate constants (k_{obs}) were obtained. In agreement with the scoring values, an increasing length of the substituent from 3 (**25**) to 4 (**26**), 6 (**27**), 8 (**28**), and 9 (**24**) carbon atoms led to an increase of $k_{obs}/[I]$ values (Figure 2C,D; Supporting Information, Figure S5). In contrast, **29** with a dodecyl substituent did not bind ClpP, as indicated by the inhibition assay and intact-protein mass spectrometry.

To further validate the suggested binding mode, we mutated several residues of the putative binding pocket and looked for changes in inhibitor binding (for characterization data on the mutants tested, see the Supporting Information, Table S3). First, we enlarged the binding pocket to allow

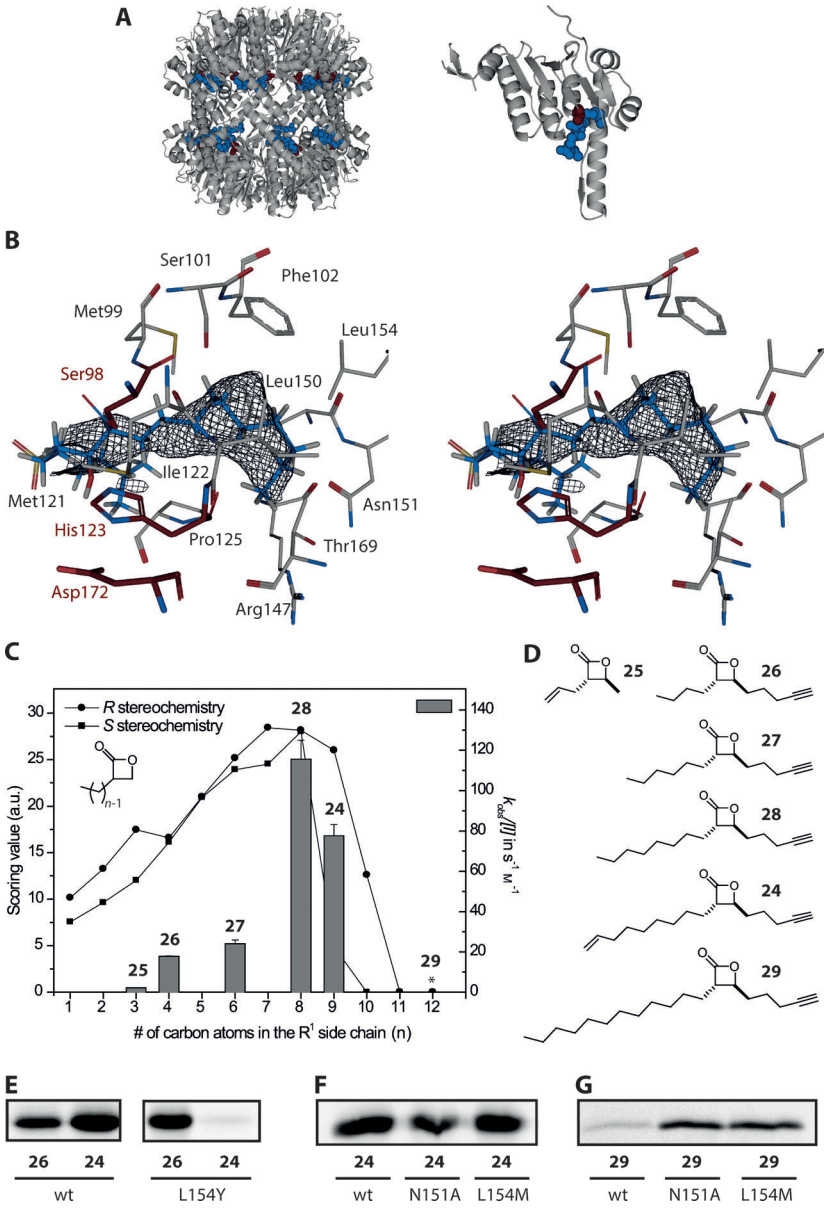


Figure 2.
A) Molecular docking of **2** (light blue) covalently bound to the catalytic serine 98 (red) in tetradecameric SaClpP (left image). Close-up view on one monomer (right image). B) Stereoview of the docking pose that reveals the presence of a hydrophobic pocket (black mesh) next to the active site (red) that accommodates the R^1 side chain (n-$C_{10}H_{21}$). C) Docking scores for virtual lactones substituted with aliphatic R^1 side chains with different lengths. $k_{obs}/[I]$ values for racemic compounds **24**–**28** as measured with recombinantly expressed SaClpP (Supporting Information, Figure S5). ⋆ denotes that compound **29** does not inhibit SaClpP. D) Structures of compounds **24**–**29** exhibiting aliphatic R^1 side chains with different lengths. E) The L154Y mutant is labeled by compound **26**, but not compound **24**, which is consistent with a smaller binding pocket. F,G) Wild-type enzyme reacts with probe **24**, but not with probe **29**. Mutant proteins N151A and L154M with an artificially enlarged binding pocket are labeled by both probes **24** and **29**. (See the Supporting Information, Figure S4 B–D, for loading controls.)

accommodation of **29**. Replacement of Asn154 at the rear end of the binding pocket by alanine as well as substitution of Leu154 by a linear and more flexible methionine enabled binding of **29** (Figure 2 E,F). Conversely, a ClpP enzyme in which Leu154 is replaced by a sterically more demanding tyrosine did not bind compound **24** while binding of smaller compound **26** was still possible (Figure 2 G). These data confirm the excellent correlation between calculated modeling predictions and experimental results.

According to the suggested binding mode the R^1 side chain of β-lactones is oriented into the hydrophobic pocket and thus contributes significantly to the observed binding affinity. To further validate the importance of this substituent, we synthesized analogous β-lactones with only one substituent at either R^1 or R^2 position. In line with the prediction, racemic lactone **30** with a decyl group at the R^1 position inhibited SaClpP, while no inhibition could be observed for racemic lactone **31** carrying a decyl group at the R^2 position (Figure 3 A). Longer incubation times up to 24 h and higher excess of inhibitor (up to 1000-fold) did not lead to binding of **31** as investigated by mass spectrometry (see the Supporting Information, Figure S6 A for further details).

To translate our findings into the design of ligands that fill up the hydrophobic pocket, we applied a computational rescaffolding for R^1 to identify putative substituents. We repeatedly found that aromatic rings were placed into the pocket; we therefore synthesized **32** with a phenylethyl group at the R^1 position. In line with the rescaffolding, **32** turned out to be a potent SaClpP inhibitor (Figure 3 B). As expected, **33** with the phenylethyl group at the R^2 position did not show inhibition.

In search of the structural basis of the observed stereochemical preference of the ClpP protease, we performed a non-covalent docking mimicking the binding situation before formation of the acyl–enzyme complex (Supporting Information, Figure S6 B). All of the compounds could be docked with the lactone moiety being placed near the catalytic Ser98. We then compared the binding modes of representative enantiomeric pairs of β-lactones (Figure 3 F; Supporting Information, Figure S7). We noted that the *S*,*S*-isomers show better scoring values compared to the respective *R*,*R*-isomers in most cases (Supporting Information, Table S2). Moreover, the carbonyl groups of *S*,*S*-configured lactones were positioned towards the oxyanion hole, while carbonyl groups of *R*,*R*-configured lactones were pointing into the opposite direction in order to enable placement of the R^1 substituent into the hydrophobic pocket (Figure 3 F). This steric consideration presumably leads to a less favored positioning of *R*,*R*-configured lactones for nucleophilic attack by Ser98, in

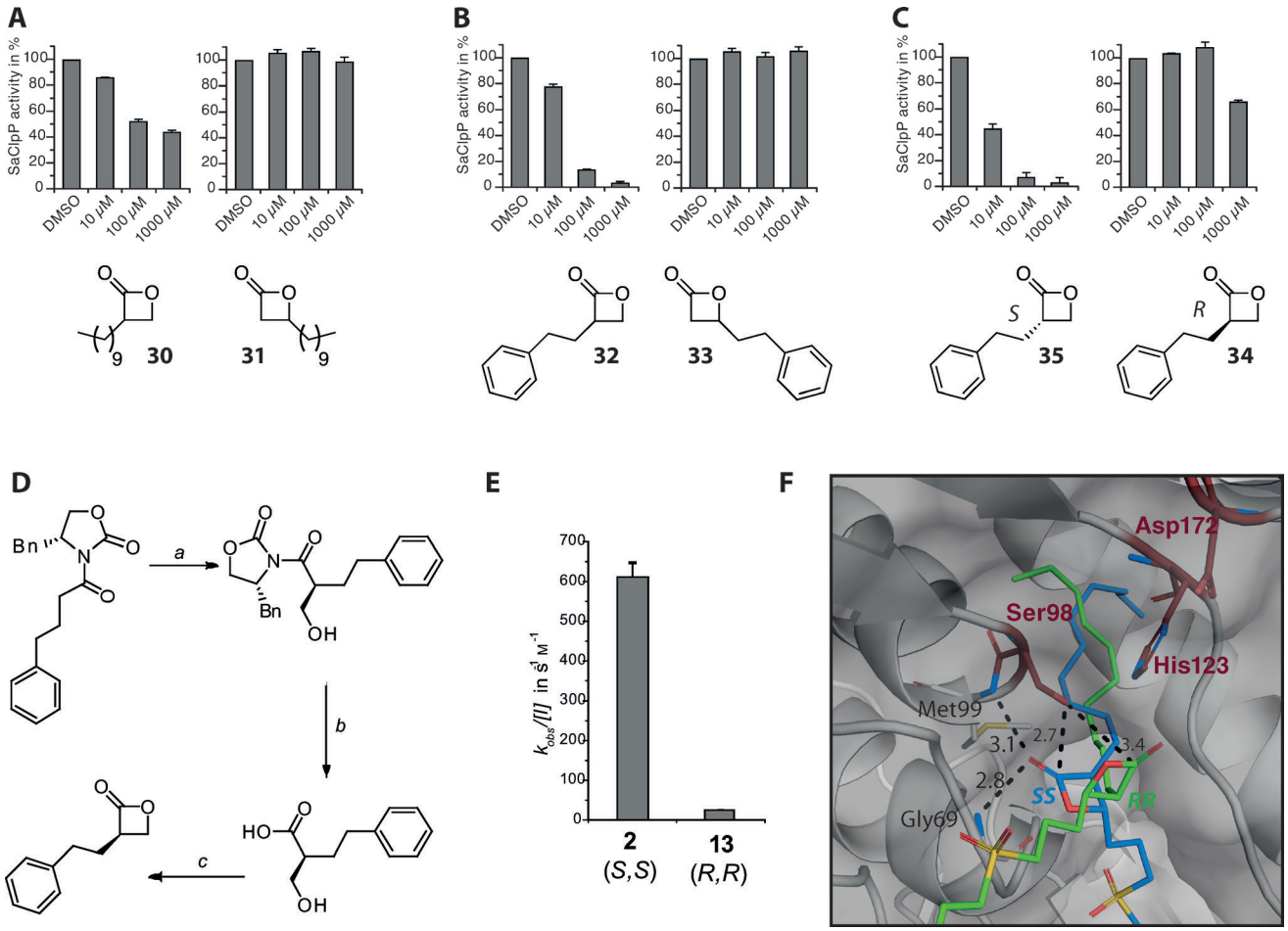
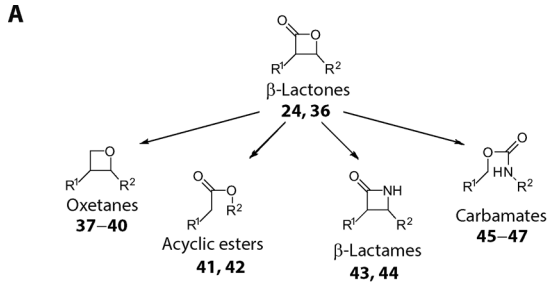


Figure 3. A–C) Compound inhibition data for β-lactones **30**–**35** emphasizing the preference of SaClpP for R^1-substituted β-lactones with *S* configuration. D) Synthesis of enantiopure β-lactones **34** and **35**. a) $(CH_2O)_x$, $TiCl_4$, iPr_2EtN, CH_2Cl_2, 50%; b) LiOH, H_2O_2, THF, H_2O, 86%; c) HBTU (*O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate), iPr_2EtN, CH_2Cl_2, 9%. E) $k_{obs}/[I]$ values for enantiomeric compounds **2** and **13** (for structures see Figure 1 E). F) Non-covalent docking of *S*,*S*-configured β-lactone **2**, revealing binding into the active site (red) with optimal geometry for attack of the active site serine (2.7 Å). The carbonyl group of **2** (blue) is accommodated in the oxyanion hole built up by amide backbone groups of Glu69 and Met99. Enantiomeric lactone **13** (green) points into opposite direction, thus likely explaining its lower binding kinetics (see Figure 3 E).

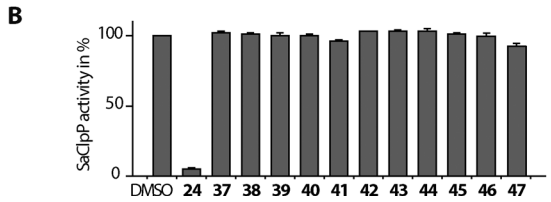
agreement with the predicted docking poses. The higher reactivity of *S*,*S*-lactones as represented by their approximately 25-fold higher $k_{obs}/[I]$ value (Figure 3 E) is most likely a consequence of the better suited stereochemistry to address the oxyanion hole. To test if this also applies to monosubstituted lactones, we synthesized enantiopure versions of **32** by a chiral-auxiliary-supported aldol reaction (Figure 3 D). As predicted by modeling, *R*-configured **34** did not inhibit ClpP, while *S*-configured **35** led to concentration dependent inhibition (Figure 3 C) and covalent modification of the active sites in all 14 subunits. Consistently, enantiopure *S*-configured **35** was approximately twice as active as racemic **32**.

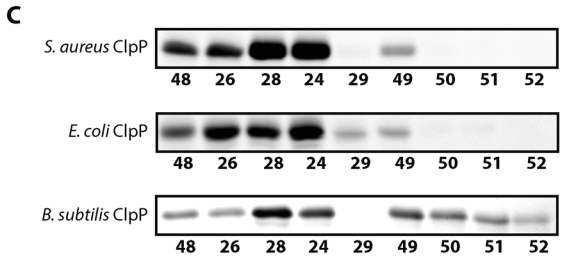
To date, β-lactones are the only non-peptidic inhibitor scaffold that exhibits specificity for ClpP, raising the question if other core structures could substitute and retain activity. We therefore utilized our knowledge on ligands **24** and **36** that satisfy the binding into the hydrophobic pocket and appended these substituents on other core scaffolds. We synthesized derivatives **37**–**40** in which the electrophilic β-lactone was replaced by an unreactive but geometrically closely related oxetane moiety (Figure 4 A,B). However, no blockage of SaClpP function was observed up to 1 mM for racemic mixtures of both *trans*- and *cis*-configured oxetanes. As β-lactones are cyclic esters, we also synthesized acyclic esters **41** and **42**. However, evaluation by peptidase assay did not reveal them as ClpP inhibitors. Although several carbamates are reported as potent inhibitors of serine hydrolases, carbamates **45**–**47** were not successful as inhibitors. Interestingly, even a β-lactam unit in **43** and **44**, the closest mimetic possible, did not result in any inhibitory effect. These results emphasize the unique reactivity and geometry of β-lactones for mechanism-based inhibition of ClpP.

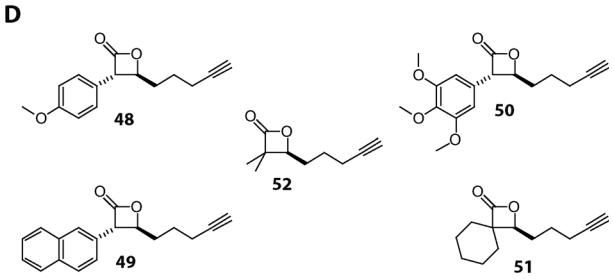
We were also curious as to whether other members of the ClpP family of proteins can be inhibited. We cloned, expressed, and purified the ClpP proteins from *Escherichia coli* (EcClpP) and *Bacillus subtilis* (BsClpP), which share 64% and 79% sequence identity and 76% and 85% sequence similarity to SaClpP. In both enzymes, the binding pockets



Core	Number	R^1	R^2	Stereo
β-lactone	**24**	$(CH_2)_7CH{=}CH_2$	$(CH_2)_3C{\equiv}CH$	*trans*
	36	$(CH_2)_7CH{=}CH_2$	$(CH_2)_2Ph$	*trans*
oxetane	**37**	$(CH_2)_7CH{=}CH_2$	$(CH_2)_2Ph$	*cis*
	38	$(CH_2)_7CH{=}CH_2$	$(CH_2)_2Ph$	*trans*
	39	$(CH_2)_7CH{=}CH_2$	$(CH_2)_3C{\equiv}CH$	*cis*
	40	$(CH_2)_7CH{=}CH_2$	$(CH_2)_3C{\equiv}CH$	*trans*
ester	**41**	$(CH_2)_7CH{=}CH_2$	Me	-
	42	$(CH_2)_7CH{=}CH_2$	^{t}But	-
β-lactam	**43**	H	$(CH_2)_2Ph$	-
	44	$(CH_2)_2Ph$	H	-
carbamate	**45**	$(CH_2)_4C{\equiv}CH$	$(CH_2)_7CH{=}CH_2$	-
	46	$(CH_2)_4C{\equiv}CH$	$(CH_2)_4CH_3$	-
	47	$(CH_2)_4C{\equiv}CH$	$(CH_2)_2Ph$	-







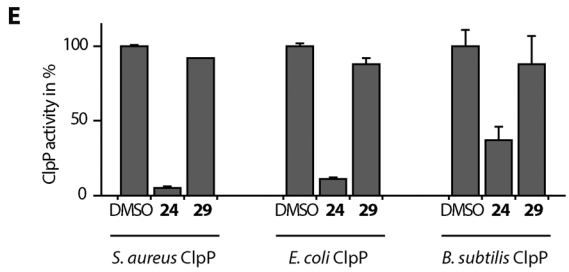


Figure 4. β-Lactones are privileged structures for ClpP inhibition. A) Core scaffolds investigated in this study. B) Residual activity of SaClpP (1 μM) after incubation with compounds **37**–**47** (100 μM) for 15 min at 32 °C. C) Recombinant ClpP enzymes from different organisms were incubated with indicated lactones (50 μM) for 1 h followed by addition of a rhodamine dye by click chemistry. (See the Supporting Information, Figure S4 E for loading controls.) D) Structures of lactones **48**–**52**. E) Compound **24** inhibits SaClpP, EcClpP, and BsClpP peptidase activity while **29** does not, thereby indicating similarly shaped binding pockets for all three organisms.

have a similar geometry to SaClpP and are predominantly hydrophobic.[3b,g] Upon labeling with a diverse set of β-lactones[2] (**24**–**29, 48**–**52**), we received similar patterns with ClpP enzymes from all three organisms (Figure 4 C,D) with BsClpP tolerating also more bulky R^1 substituents. Consequently, treatment of EcClpP and BsClpP with **24** but not with the dodecyl compound **29** corresponded to reduction of enzyme activity, as revealed by fluorogenic substrate assays.

The existence of the hydrophobic binding pocket may not only have significant impact on the design of potent inhibitors and corresponding anti-virulence pharmaceuticals but also contribute to our understanding of the natural cleavage site specificity of ClpP.

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